SAFETY ASSURANCE AND QUALITY ENHANCEMENT OF JUICES BY THE APPLICATION OF TRADITIONAL THERMAL TREATMENTS AND NONTHERMAL PROCESSES

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Outbreaks associated with the consumption of contaminated juices have stressed the relevance of introducing a microbial killing step during processing of these products. Nonetheless, some unanswered questions regarding the application of pasteurization and nonthermal technologies, such as UV light, to ensure the safety of beverages with a pH below 4.6 have been identified. Hence, this research project aimed to address some of these existing gaps of information and therefore assist the food industry, regulatory agencies and process authorities with the establishment of critical limits for the safe thermal or UV processing of low-pH drinks. The thermal tolerance of *E. coli* O157:H7 in apple-carrot juice blends, using different acids and at variable pH values was determined. Then, the effect of different methodologies of acid adaptation and acid shock on the thermal tolerance and survival of three Shiga toxin-producing *E. coli* strains was assessed. Furthermore, the impact of the concentration of insoluble solids and the darkening of juice after apple pressing, on the efficiency of UV treatments were studied and, the influence of the addition of selected additives and preservatives on the efficiency of the UV treatment of apple juice and the impact of UV radiation over those compounds was additionally evaluated. This dissertation will provide the juice and beverage industries with relevant information to meet some of the science-based rules stated in current and new regulations, including the Food Safety Modernization Act.

BIOGRAPHICAL SKETCH

A Costa Rican citizen, Jessie Usaga holds a BS degree in Food Technology from the University of Costa Rica, where she has also carried out duties as assistant professor. Jessie joined Cornell in 2011 thanks in part to a Fulbright-LASPAU scholarship and partial funding from the University of Costa Rica, and as a graduate student, she held Teaching (Product Development Course) and Extension (Northeast Center for Food Entrepreneurship, Geneva NY) Assistantships. She also served as an officer of the Student Association of the Geneva Experiment Station (SAGES). Jessie became actively involved with the Institute of Food Technologists (IFT), representing Cornell students at the Western New York Section (WNYIFT), and Latin American students at the Nonthermal Processing Division (NPD).

Jessie presented seven posters at national conferences and won 2nd and 1st places in the Graduate Research Paper Competitions (NPD) during IFT's 2012 and 2013 Annual Meetings. In 2013 she was selected finalist for the Developing Scientist Competition organized by the International Association for Food Protection (IAFP). Jessie received the WNYIFT Ph.D. Student Scholarship (2012), The Juice Products Association-Davis Scholarship (2013), IFT Feeding Tomorrow Scholarship (2013), The Kosi Award in Food Science (2014), and IAFP Student Travel Scholarship (2014). Jessie carried out teaching, research and extension responsibilities at the University of Costa Rica and upon completion of her Ph.D., she will resume her role as faculty member. She aspires to promote food safety research initiatives and to continue supporting local food companies by providing training and technical advice in food processing and safety.

DEDICATION

In dedication to my best friend and favorite labmate and colleague, my husband Óscar, who supported me even when this project was only a dream; and to my parents, the most inspiring example of hard work and dedication and whose love, encouragement and prayers accompanied me all the way through the end.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance

AS: Acid shock

ATCC: American Type Culture Collection

BPD: Butterfield's buffer phosphate diluent

CFU: Colony forming unit

DHA: Dehydroascorbic acid

DTT: Dithiothreitol

FDA: U.S. Food and Drug Administration

FSMA: Food Safety Modernization Act

MPA: Metaphosphoric acid

HACCP: Hazard analysis and critical control points

HPLC: High performance liquid chromatography

NTU: Nephelometric turbidity units

RSIN: Reflectance-specular included

SIS: Suspended insoluble solids

TSA: Trypticase soy agar

TSB: Trypticase soy broth

US: United States

UV: Ultraviolet

CHAPTER 1

INTRODUCTION AND RESEARCH OBJECTIVES

Globally, the commercialization of juice-based beverages signifies an important source of revenue for the food industry. In 2013, juices and juice drinks represented $15.5 billion of US sales and, in the past 5 years, the developing markets of Asia-Pacific and Latin America have shown strong growth rates for these products (*16*). Furthermore, in 2013, juice innovation soared by 30% worldwide, led by efforts in Europe, and France in particular (*16*). Unfortunately, outbreaks due to the consumption of contaminated and unpasteurized juices have occurred in the United States and around the world (*5*). This situation has prompted the establishment of more strict regulations to ensure the safe and sanitary processing of these products (*23*).

In 1979, when the federal regulations governing acidified foods (21 Code of Federal Regulations [CFR] part 114) were established in the United States, vegetative pathogenic microorganisms were not considered a significant biological risk for acid and acidified food products. Therefore, this regulation was primarily designed to prevent the spore outgrowth and toxin production of *Clostridium botulinum,* which does not occur if the pH is maintained at or below 4.6 (*2*). However, recent outbreaks involving the consumption of acid and acidified food products (including some juices) contaminated with foodborne pathogens such as *Salmonella* and Shiga toxin-producing *Escherichia coli,* have stressed the importance of establishing a microbial killing step during processing of these products. Moreover, studies have demonstrated that even though these pathogenic microorganisms may not grow in acid and acidified products due to

the low pH (*2*), a gradual exposure of certain pathogens, for example *E. coli* O157:H7, to moderate acidic environments may enhance their thermal tolerance and survival ability when present in low-pH products (*9*, *13*, *15*, *18, 20*, *21*), which is the case of most of fruit juices.

To prevent further outbreaks due to contaminated juices, the U.S. Food and Drug Administration has established that processors must ensure a minimum of 5-log reduction of the pertinent pathogen likely to occur in the product (FDA, 2001). This reduction is achievable by the application of unit operations that have been specifically designed and validated to kill disease-causing microorganisms. For example: a) traditional heat treatments including pasteurization and sterilization, b) nonthermal technologies such as ultraviolet (UV) light and high pressure processing or c) a combination of both, thermal and nonthermal technologies. Nowadays, the thermal treatments remain as an effective, recognized and the most commonly used approach to prevent food safety issues (*6, 14*). However, little is known about the minimal pasteurization regime (heating temperatures and processing times) required to guarantee the safety of non-shelf stable liquid food products with a pH below or equal to 4.6 and commercialized under refrigerated conditions. Consequently, for some beverages with these characteristics, the juice industry has been using overestimated heat treatments that may have a negative effect on the quality and nutritional properties of the products. Additionally, only a few studies have been published regarding the thermal tolerance of enterohemorrhagic *E. coli* in acidic juices and similar liquid food products such as sauces and dressings with a wide range of low pH values adjusted by adding different acidulants. Likewise, information regarding the appropriate

methodology to conduct challenge microbiological studies in this type of products is limited. Thus, the elucidation of this information is important not only for the juice and beverages industries but also for regulatory agencies and process authorities.

Regarding the application of heat treatments, the major disadvantage of this traditional approach is that, as previous research studies have shown, some adverse effects in color, flavor, and nutritional content of juices may occur due to heat exposure (*7, 17*). Changes in the quality of beverages represent a challenge for the food industry, especially considering the increased consumer demand for more fresh-like products with enhanced nutritional properties. Thus, the application of nonthermal affordable treatments such as UV light has attracted the interest of the juice industry, especially for small and medium sized juice producers in the United States. Since 2000, the FDA has recognized UV light as a nonthermal-processing alternative to pasteurization (*24*). This low-cost technology (*4, 10*) has been proven effective against pathogens (*1, 8, 19*), and has been associated with limited changes in quality parameters (*3, 22*). However, its application is restricted because, as reported in the literature, the colored compounds and insoluble solids present in some juices may absorb UV light and therefore reduce the antimicrobial capacity of the technology (*11, 12*). Worth noting, most of the research that has been previously performed to address the effect of the concentration of solids and colored compounds on the efficiency of the technology was executed using a laboratory scale UV machine that operates under a laminar flow regime. Thus, considering that the FDA has stated on the regulations that turbulent flow rate must be ensured for the UV treatment of juices, it becomes relevant to evaluate these effects while ensuring the recommended processing conditions.

This research project aims to address some of the mentioned unanswered questions and existing gaps in the literature regarding the application of thermal pasteurization and UV light treatments of low-pH juices. The goal of the study is to provide the juice and beverage industries with relevant information to meet the science-based rules stated on current and new regulations including the FDA Food Safety Modernization Act (FSMA) and the Acidified Foods Draft Guidance (*25*). Hence, the following objectives have been established:

Objective 1: Determine the effect of varying the pH of an apple-carrot juice blend, by adding different organic acidulants, on the thermal tolerance parameters of acid adapted and unadapted *E. coli* O157:H7 ATCC C7927.

Objective 2: Evaluate the effect of acid adaptation and acid shock on the thermal tolerance and survival of *E. coli* O157:H7 (strains C7927 and ATCC 43895) and *E. coli* O111 in apple juice.

Objective 3: Determine the effects of the concentration of suspended insoluble solids and time after apple pressing on the efficiency of UV treatment of cloudy apple juice.

Objective 4: Assess the effect of the addition of ascorbic acid and selected preservatives on the efficiency of the UV treatment of apple juice, and the effect of the UV exposure over those compounds.

Objective 5: Determine the appropriate frequency of revalidation for a commercial UV processing unit.

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CHAPTER 2

THERMAL RESISTANCE PARAMETERS OF ACID-ADAPTED AND UNADAPTED *ESCHERICHIA COLI* O157:H7 IN APPLE CARROT JUICE BLENDS: EFFECT OF ORGANIC ACIDS AND PH[1]

ABSTRACT

Numerous outbreaks involving fresh juices contaminated with *Escherichia coli* O157:H7 have occurred in the US and around the world, raising the concern for the safety of these products. Until now only a few studies regarding the thermal tolerance of this pathogen in acidic juices over a wide range of pH values have been published. Therefore, the effect of varying the pH with different organic acids on the thermal inactivation of non-acid adapted and acid-adapted *E. coli* O157:H7 (strain C7927) was determined. The decimal reduction times (*D*-values) and the change in temperature required for the thermal destruction curve to traverse one log cycle (*z*-values) were calculated for non-acid adapted *E. coli* in an apple-carrot juice blend (80:20) adjusted to three pH values (3.3, 3.5, and 3.7) by addition of lactic, malic, or acetic acid, and at a pH of 4.5 adjusted with NaOH. Thermal parameters were also determined for acid-adapted cells in juices acidified with malic acid. The effect of soluble solids content on the thermal tolerance was studied in samples with a pH of 3.7 at 9.4 to 11.5°Brix. The *D*-values were determined at 54, 56, and 58°C, and trials were conducted in triplicate. Non-acid-adapted *E. coli* exhibited the highest thermal tolerance at pH 4.5 (*D*-value at 54°C [$D_{54°C}$] of 20 ± 4 min and *z*-value of 6.2°C), although on average the *D*-values increased significantly ($P < 0.01$) due to acid adaptation. In acidified juices, the highest tolerance was observed in acid-adapted *E. coli* in samples adjusted to pH 3.7 with malic

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acid ($D_{54°C}$ of 9 ± 2 min and *z*-value of 5.4°C), and the lowest in unadapted *E. coli* at pH 3.3 acidified with acetic acid ($D_{58°C}$ of 0.03 ± 0.01 min and *z*-value of 10.4°C). For juices acidified to the same endpoint pH with different acids, *E. coli* was found more tolerant in samples acidified with malic acid, followed by lactic, and acetic. Increasing the soluble solids content from 9.4 to 11.5°Brix showed no significant effect on the thermal tolerance of *E. coli* ($P > 0.01$). Data from this study will be useful for establishing critical limits for safe thermal processing of pH controlled juices and similar products.

INTRODUCTION

Outbreaks involving the consumption of unpasteurized acidic juices such as apple juice and cider contaminated with enterohemorrhagic *Escherichia coli* O157:H7 have been reported (*11*). Consequently, juice safety represents a public health concern and has prompted the establishment of regulations to ensure a safe and sanitary processing of these products (*35*). The U.S. Food and Drug Administration has established that juice processors must ensure a minimum of 5-log reduction of the most resistant pathogen likely to occur in the product (*35*). Traditional thermal treatments remain an effective, recognized and commonly used approach for fulfilling this requirement (*16*, *25*). However, thermal pasteurization is not sufficient for destroying heat resistant spores and therefore, acidification has been commonly applied by the food industry as a pre-treatment to pasteurization to inhibit spore germination and to allow milder time and temperature conditions during heat treatments (*12, 15*).

Previous research has shown that the ability of *E. coli* O157:H7 to survive in acidified food products is of concern because the resistance of this foodborne pathogen to

environmental stress increases when the microorganism is gradually exposed to moderately acidic environments (*18, 22*). This represents a further challenge when a safe thermal process needs to be established for pH-controlled products, including acidic and acidified beverages.

Juices' intrinsic and extrinsic properties, including the pH and organic acid composition, soluble solids content, addition of preservatives and other ingredients with antimicrobial properties, represent some of the variables that may play a predominant role in the heat tolerance and survival response of *E. coli* O157:H7 (*13*, *28, 31, 34, 37*). Limited studies exist on the impact of varying pH and using organic acidulants on the heat resistance of *E. coli* O157:H7 strains (particularly strains isolated from acid-juice-associated outbreaks).

Prior to this study, Hsiao and Siebert (*17*) postulated a mathematical model to predict the inhibitory effect of organic acids on bacterial growth. Their model was established using non-pathogenic surrogates, including *E. coli* ATCC 25922, and its validation for pathogenic strains has not been executed yet. Gabriel (*16*) also published a comprehensive study that evaluated the influences of various combinations of process and product parameters including heating temperature (53 to 62°C), pH (2.0 to 7.0) and soluble solids content (1.4 to 69°Brix), on the thermal inactivation of non-acid-adapted and acid-adapted *E. coli* O157:H7 (HCIPH 96055) in a liquid heating medium. A robust model was developed and its validation was further performed using different fruit juices. Nonetheless, since previous studies have revealed significant differences in the heat tolerance among *E. coli* O157:H7 strains (*14, 25, 33*) as well as variations on the

survival response in acidic environments even within a single serotype (*4*), the use of the suggested model might be limited for certain strains. Moreover, the validation of this model using other enterohemorrhagic *E. coli* strains isolated from acid and acidified food products should be performed.

The objective of this study was to evaluate the effect of pH variation by the addition of different organic acidulants commonly used by the beverage industry on the thermal inactivation of acid-adapted and unadapted *E. coli* O157:H7 (strain C7927) in an apple-carrot juice blend, representing a turbid fruit juice blend with added nutrients and suspended insoluble solids due to the 20% carrot juice formulation. Both apple and carrot juices have been implicated in foodborne outbreaks (*2, 6, 30*).

MATERIALS AND METHODS

Juice blend extraction. An 80% apple and 20% carrot juice blend was prepared using carrots purchased from a local supermarket and stock apples from controlled atmosphere (8 to 12 months) storage at the New York State Agricultural Experiment Station (Geneva, NY). Due to storage time, visually sound apples were hand-selected from apples exhibiting mold and/or rot. Fruits and roots were hand washed in cold water to remove visible soil and carrot top remnants. Unpeeled apples and carrots were passed through a commercial food processor (Robot Coupe USA. Inc., Ridgeland, MS), and pressed in a hydraulic rack-and-frame press (Loomis Engineering & MFG Company, Caldwell, NJ) for juice extraction. The resulting juice blend was not sterilized prior to inoculation to prevent heat precipitation of carrot solids and to represent the

microbial load of a fresh-pressed juice. To prevent fermentation, juice was stored at -23°C until used.

Physicochemical characterization. pH was measured using a Thermo Scientific Orion 2 Star pH meter (Thermo Fisher Scientific, Beverly, MA). The soluble solids content were estimated with a Leica Auto Abbe refractometer model 10500-802 (Leica Inc., Buffalo, NY). Total titratable acidity was determined using a G20 compact titrator (Mettler Toledo, Schwerzenbach, Switzerland), and turbidity measurements were performed with a HACH 2100P portable turbidimeter (Hach Company, Loveland, CO). All physicochemical analyses were performed in triplicate.

Bacterial strains and media. A single isolated colony of *E. coli* O157:H7 (strain C7927), obtained from the Food Microbiology Laboratory at the New York State Agricultural Experiment Station (Geneva, NY) and originally isolated from a patient who had consumed contaminated apple cider associated with an outbreak (*34*), was transferred into 10 ml of Trypticase soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD) and incubated for 20 ± 2 h at 35 ± 2°C (to stationary-phase) on an Innova 2300 rotatory platform shaker (New Brunswick Scientific Co., Edison, NJ) at 250 rpm.

Acid-adaptation was conducted according to the protocol stated by Enache et al. (*14*), where a loopful of stationary-phase *E. coli* was transferred to Trypticase soy broth (pH adjusted at 5.0 with 1 N HCl) and incubated overnight (20 ± 2 h) at 35 ± 2°C. After incubation, 1 ml of the culture was centrifuged and the pellet was resuspended in refrigerated 0.1 M citrate buffer (pH 4.0) and stored at 4 ± 1°C for 18 h before use.

Heat tolerance determination. Thermal resistance parameters of *E. coli* O157:H7, specifically decimal reduction times (*D*-values) and the increment in temperature required to decrease the *D*-value in one log cycle (*z*-values), were determined following the methodology reported by Splittstoesser et al. (*34*) with the exception that samples were enumerated using the pour plate technique on Trypticase soy agar (TSA) (Difco, BD) instead of plate count agar, and colonies were counted after 20 ± 2 h of incubation at 35 ± 2°C, instead of 4 incubation days at 37°C. Amounts of 9 ml of the apple-carrot juice blend were dispensed aseptically in a sterile centrifuged tube and inoculated with 1 ml of the *E. coli* O157:H7 culture resulting in an initial population of 10^7-10^8 CFU·ml^{-1}. Amounts of 20 µl of inoculated juice were injected into five replicate glass melting point capillary tubes (1.5 to 1.8 by 100 mm; Kimble Chase, Vineland, NJ) using a 1 ml syringe equipped with a repeater dispenser (Hamilton Co., Reno, NV). The capillary tubes were flame sealed and immediately submerged in water test tubes contained in a stirred water bath at temperatures and times selected to obtain thermal death time curves with at least five sampling points, extended for over 4-log reductions, and showing a coefficient of determination (r^2) greater than 0.9. The range of time intervals differed depending on the tested temperature, pH of samples, and acid-adaptation of the culture. Nonheated controls were included, which corresponds to time zero in the thermal death curves. After thermal treatment, capillaries were rapidly cooled in an ice bath and then submerged in test tubes containing 70% cold ethanol to decontaminate the exterior of the capillary tubes. The five capillaries were removed from the ethanol solution, blotted to remove excess ethanol, and then transferred into a milk dilution bottle containing 20 ml of 0.1% sterile peptone water, and crushed with a sterile glass rod. Appropriate serial dilutions in sterile 0.1% peptone water were aseptically plated by

duplicate in petri dishes where 20 ml of TSA was pour plated and mixed thoroughly. After agar solidification, petri dishes were incubated and colonies counted after 20 ± 2 h of incubation at 35 ± 2°C. The *D*-values were calculated as the inverse negative value of the slope obtained from plotting the log number of survivors against the sampling time. The *z*-values were defined as the negative inverse slope of the linear regression line for the log of the calculated *D*-values over the range of tested temperatures (54, 56 and 58°C).

Influence of pH, organic acid and soluble solids content on thermal tolerance. The *D*-values for non-acid-adapted stationary-phase *E. coli* were determined in the model apple-carrot juice blend following a complete factorial design, with three levels for temperature (54, 56 and 58°C), three acids (malic, acetic, and lactic) and three pH values (3.3, 3.5, and 3.7). The acids were purchased from Fisher Scientific (Fair Lawn, NJ). The *z*-values were calculated accordingly. Heat inactivation tests were also carried out in a juice sample with a pH of 4.5 adjusted by addition of 1 N NaOH. Thermal resistance parameters were determined following the methodology indicated in "Heat tolerance determination" above. The soluble solids content of all samples were determined.

To examine whether the concentration of soluble solids had a significant influence on the heat tolerance of the strain studied, heat inactivation tests were carried out for non-acid-adapted *E. coli* in a juice sample with a pH of 3.7 adjusted by the addition of malic acid at different concentrations. Thermal parameters were determined following a factorial design with three levels for temperature (54, 56 and 58°C) and three levels for

ºBrix (11.5, 10.7, and 9.4). The pH and organic acid selected for this subsection of the study correspond to the conditions that were expected to show the highest *D*-values on the full factorial design proposed to asses the effect of pH and the acidulant on the heat tolerance of this microorganism. All *D*-values were determined from three independent experiments and *z*-values were calculated accordingly.

Influence of acid adaptation on thermal tolerance. Samples of the apple-carrot juice blend were adjusted to pH of 3.7, 3.5, and 3.3 by the addition of malic acid at different concentrations, and to pH of 4.5 with a solution of 1 N NaOH. Juices were inoculated with acid-adapted *E. coli* O157:H7 C7927 and decimal reduction times were determined from three independent experiments at 54, 56 and 58°C. *z*-values were additionally calculated. *D* and *z*-values were determined following the methodology indicated in "Heat tolerance determination" above.

To compare the heat tolerance (in terms of *D*-value) of non-acid-adapted and acid-adapted *E. coli*, a factorial design with three levels for temperature (54, 56 and 58°C), four levels for pH (3.7, 3.5, 3.3 and 4.5) and two levels for cell status (acid-adapted and unadapted) was followed.

Statistical analyses. Two- and three-way analyses of variance (ANOVA) and Tukey's honestly significant difference (HSD) test for means comparison were performed using JMP® version 10 (SAS Institute Inc., Cary, NC). Differences were considered significant at P value of < 0.01.

RESULTS AND DISCUSSION

The model juice had a pH of 4.1, total soluble solids content of 12.2°Brix, total titratable acidity of 0.33% (grams of malic acid per 100 grams of juice), and a turbidity value of 2230 nephelometric turbidity units, representing a turbid fruit-vegetable juice blend.

Influence of soluble solids content on thermal tolerance. As a result of pH adjustment, the samples used to study the effect of varying pH by adding one of three organic acids (malic, lactic and acetic) on the thermal tolerance of *E. coli* O157:H7 C7927 ranged between 9.05 and 12°Brix in the concentration of soluble solids. The average values for total soluble solids content are given in Table 1.

Table 1. Total soluble solids content of samples used to determine the *D* and *z* values of non-acid-adapted *E. coli* O157:H7 C7927 in apple-carrot juice blend.[a]

pH	Total soluble solids content (°Brix)		
	Lactic acid	Malic acid	Acetic acid
3.7	9.07 ± 0.02	11 ± 2	10.7 ± 0.3
3.5	10.6 ± 0.3	10.5 ± 0.2	11 ± 1
3.3	10.6 ± 0.1	10.9 ± 0.3	11.6 ± 0.4

[a] Values are the average ± standard deviation (n = 3).

To examine whether the reported variation of soluble solids content had a significant effect on the *D*-values of *E. coli*, the thermal parameters were determined in the apple-carrot juice blend with a pH of 3.7 adjusted by the addition of malic acid. The *D* and z values obtained are summarized in Table 2. After a natural log transformation of the *D*-values, two-way ANOVA showed a nonsignificant interaction of temperature and soluble solids content (P = 0.74). After removing the interaction term, an expected significant

effect of temperature on the *D*-values was observed ($P < 0.0001$), but soluble solids (in the tested range) were shown not to have a significant effect on the *D*-values ($P > 0.064$). The resulting model presented a coefficient of determination (r^2) of 84%. The influence of temperature on *D*-values has been extensively reported in the literature and is based on the alteration of microbial cell structures and denaturation of metabolic enzymes that adversely affect several biological processes, leading to cell death (*16, 19*). The nonsignificant effect of soluble solids on thermal tolerance of this foodborne pathogen has also been previously reported in similar food products and over the range of soluble solids studied in this investigation. Sharma et al. (*33*) for example, demonstrated a nonsignificant correlation between soluble solids content (ranging between 7.6 and 13.2°Brix) and the thermal tolerance of *E. coli* O157:H7 (EO139 and SEA 13B88) determined in cantaloupe and watermelon juices. Similarly, Splittstoesser et al. (*34*) reported a nonsignificant alteration of the *D*-values of *E. coli* O157:H7 (same strain used in this study) when soluble solids were augmented from 11.8 to 16.5ºBrix in a single-strength Empire apple juice.

Table 2. *D* and *z*-values of non-acid-adapted *E. coli* O157:H7 C7927 in apple-carrot juice blend (pH adjusted to 3.7 with malic acid) with three total soluble solids content.[a]

ºBrix	*D*-value (min) at given temperature (°C)			*z* value (°C)
	54	56	58	
11.5	4.3 ± 0.7	3 ± 1	1.1 ± 0.3	6.6
10.7	6 ± 2	3.1 ± 0.3	1.4 ± 0.7	6.3
9.4	3.7 ± 0.4	2.7 ± 0.9	1.1 ± 0.4	7.7

[a] Values are the average ± standard deviation (n = 3).

Table 3. *D* and *z*-values of non-acid-adapted *E. coli* O157:H7 C7927 in apple-carrot juice blend, acidified at three pH values with three organic acids (average ± standard deviation for n = 3).[a]

pH	*D*-value (min) at 54°C			*D*-value (min) at 56°C			*D*-value (min) at 58°C			*z*-value (°C)		
	Lactic	Malic	Acetic	Lactic	Malic	Acetic	Lactic	Malic	Acetic	Lactic	Malic	Acetic
3.7	1.87 ± 0.03^{C}	6 ± 1^{A}	1.48 ± 0.05^{C}	1.2 ± 0.3^{IJ}	2.94 ± 0.02^{H}	0.7 ± 0.1^{J}	0.40 ± 0.07^{W}	2.1 ± 0.3^{U}	0.5 ± 0.1^{W}	6.0	9.1	8.1
3.5	0.66 ± 0.04^{D}	3.0 ± 0.3^{B}	0.26 ± 0.01^{E}	0.4 ± 0.1^{K}	1.7 ± 0.4^{I}	0.22 ± 0.05^{L}	0.207 ± 0.001^{X}	1.1 ± 0.1^{V}	0.10 ± 0.01^{Y}	8.0	9.2	9.2
3.3	0.134 ± 0.003^{F}	1.9 ± 0.5^{C}	0.08 ± 0.02^{G}	0.086 ± 0.004^{M}	1.0 ± 0.1^{J}	0.049 ± 0.004^{N}	0.08 ± 0.02^{Y}	0.5 ± 0.1^{W}	0.03 ± 0.01^{Z}	16.7	6.9	10.4

[a] Values in the same temperature quadrant not sharing a common superscript letter represent significantly different values ($P < 0.01$) based on post hoc multiple comparisons with a Tukey correction following a two-way ANOVA run on a log transformed response.

Influence of varying pH and organic acid on thermal tolerance. The *D* and *z*-values of stationary-phase (non-acid-adapted) *E. coli* O157:H7 C7927 are presented in Table 3. Figure 1 shows a representative thermal death time curve determined at 54°C for juice adjusted at three pH values (3.7, 3.5 and 3.3) by addition of acetic acid. Similar curves were obtained for all conditions tested and were used to calculate the *D*-values.

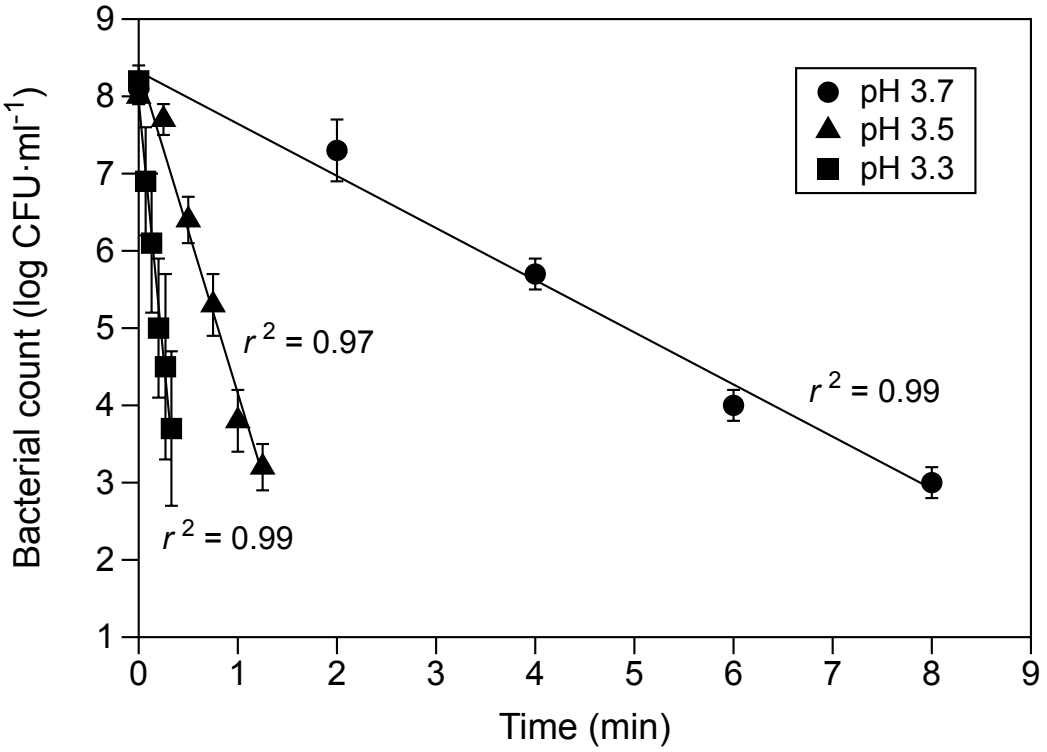

Figure 1. Representative thermal death time curve for non-acid adapted *E. coli* O157:H7 C7927 in apple-carrot juice blend at 54°C, adjusted at three pH values by addition of acetic acid. Error bars represent standard deviation for n = 3.

After a natural log transformation of the *D*-values, three-way ANOVA showed a significant triple interaction between temperature, acid and pH (P = 0.0002). Therefore, data was further analyzed by performing three independent two-way ANOVAs for each temperature (54, 56 and 58°C) with two factors, acid and pH,

at three levels each. The three resulting models showed coefficients of determination (r^2) of 99% and a consistent significant interaction between pH and acid ($P < 0.0001$).

For samples acidified to the same endpoint pH and regardless of the tested acidulant, *E. coli* was found more tolerant in juices acidified with malic acid, followed by lactic, and acetic acids, except when the *D*-value was determined at a pH of 3.7, the highest of the three acidified pH values, where no significant differences were detected in samples acidified with lactic and acetic acids, regardless of the tested temperature ($P > 0.01$). The toxicity of organic acids to bacterial cells is attributed to a lowering cytoplasmic pH and intracellular accumulation of acid anions (*20*), and the antimicrobial effect depends upon the organic acid's pK_a value and the pH of the external medium. Theoretically, lactic acid (pK_a 3.86) is a stronger acid when compared to acetic (pK_a 4.79) (*1*) and malic acid (pK_{a1} 3.40, pK_{a2} 5.20) (*17, 21*). However, conflicting results regarding the effect of different organic acidulants on bacterial inactivation have been reported in the literature. For example, Buchanan and Edelson (*4*) found that at a pH of 3.0 and at 37°C, lactic acid was the most deleterious acidulant for seven enterohemorrhagic *E. coli* strains, in comparison with acetic and malic acids. In agreement with this, Cheng et al. (*8*) found that lactic acid was more lethal than acetic acid for acid-adapted and non-acid-adapted *E. coli* O157:H7 ATCC 43889 in a saline solution acidified to a pH of 3. Contradictorily, and in agreement with our findings, Ryu et al. (*32*) stated that acetic acid was the most lethal acidulant

to *E. coli* O157:H7 (strain E0139), followed by lactic and malic acids, when tested over a pH range from 3.9 to 5.4 and at 37°C. According to Bjornsdottir et al. (*3*), the reported differences may result from variations in the experimental design and methodologies used to assess effects of acidification. In addition, factors such as temperature, pH, acid concentration and ionic strength, as well as the bacterial strain and environmental features including growth phase and induced acid resistance, may affect the antimicrobial activity of organic acids (*3*).

Regarding the effect of the pH within the same heating temperature, longer exposure times were needed for inactivation of the test strain as pH increased. This result agrees with findings reported by Chikthimmah (*10*), where increasing the pH of cider (from 3.2 to 4.7) decreased the rate of destruction of *E. coli* O157:H7 (SEA 13889).

The *D*-values were additionally determined for the same strain at a pH of 4.5, obtaining a *D*-value at 54°C ($D_{54°C}$) of 20 ± 4, a $D_{56°C}$ of 9 ± 2, and a $D_{58°C}$ of 4.5 ± 0.6 (with a *z*-value of 6.2°C), corresponding to the highest thermal tolerance exhibited by the non-acid-adapted *E. coli* O157:H7 C7927 strain. Concerning the *z*-values, no consistent trends were observed for the three organic acids and pH values evaluated in this study. Published investigations regarding the influence of pH on the *z*-values of different foodborne pathogens and spoilage microorganisms have shown important differences and even contradictory results (*29*). Accordingly, some authors have found higher *z*-values

when the pH was diminished while the opposite trend has also been reported (*5, 7, 23, 26*). Other researchers, ourselves included, have been unable to identify the influence of pH over this thermal resistance parameter (*24*) and therefore, further investigation is required to identify the sources of the reported differences.

Influence of acid adaptation on thermal tolerance. The *D* and *z*-values for acid-adapted *E. coli* O157:H7 C7927 determined at pH values of 3.3, 3.5 and 3.7 (adjusted by the addition of malic acid), and at a pH of 4.5 (adjusted with 1 N NaOH) are presented in Table 4. These values were compared to those obtained from the non-acid-adapted cells (reported in the previous section and Table 3), and after a natural log transformation of the *D*-values, three-way ANOVA showed a nonsignificant interaction between temperature, pH and cell status ($P = 0.03$). After removing the three-way interaction term, the three resulting two-way interactions were deemed nonsignificant ($P > 0.01$). After removal of these interactions from the model, it was found that, on average, acid adaptation of *E. coli* O157:H7 C7927 significantly increased the *D*-values ($P = 0.0003$). As expected, increments in pH ($P < 0.0001$) and reduction in the heating temperature ($P < 0.0001$) lead to significantly greater *D*-values. After post hoc multiple comparisons with a Tukey correction, these values were deemed significantly different from each other at all tested temperatures and pH values ($P < 0.01$). The model's coefficient of determination (r^2) was 92%. These latter findings are consistent with the trends that we previously reported for the non-acid-adapted *E. coli* subjected to the same experimental conditions.

Table 4. *D* and *z*-values of acid-adapted *E. coli* O157:H7 C7927 in apple-carrot juice blend, adjusted at four pH values with malic acid and NaOH.[a]

pH	*D*-value (min) at given temperature (°C)			*z* value (°C)
	54	56	58	
4.5	14 ± 4	8 ± 2	5 ± 2	8.6
3.7	9 ± 2	5 ± 1	1.7 ± 0.4	5.4
3.5	5.6 ± 0.6	2.6 ± 0.9	1.2 ± 0.1	5.9
3.3	3.1 ± 0.2	1.6 ± 0.9	0.8 ± 0.4	6.8

[a] Values are the average ± standard deviation (n = 3).

Previous studies have demonstrated that heat resistance of stationary-phase cells of several Shiga toxin-producing *E. coli* strains is generally equal to or higher than their acid adapted counterparts. This could be explained by a cross-protection effect on cell membranes (*9, 27*) that potentially enhances bacterial tolerance to different sources of stress such as heat, ionizing and non-ionizing irradiation (*4*). Stationary phase and starvation induced protective proteins in *E. coli* that are regulated by σ^s (*rpoS*) and impart resistance to chemical and physical changes (*9)*. It was previously suggested that these *rpoS*-regulated proteins sustained acid tolerance of *E. coli* O157:H7 and enhanced the pathogen's tolerance and survival in fermented sausage (*9*).

Interestingly, despite the fact that our methodology for determination of *D*-values differed from the protocol used by Enache et al. (*14*), and that this study was performed on a juice blend rather than on a single-strength apple juice, we found that at a pH of 3.7 and 56°C, the *D*-value obtained for acid-adapted *E. coli* O157:H7 C7927 was not noticeably different from the value reported for *E. coli*

O157:H7 strain N-4073 (*14*). Moreover, the *D*-values obtained in this study were consistently higher than *D*-values reported by Enache and co-workers (*14*) for non-O157:H7 strains, including the serogroups O26, O45, O103, O111, O121 and O145.

When applying the predictive model proposed by Gabriel (*16*) to calculate the expected *D*-values for acid adapted *E. coli* O157:H7 C7927 under all the conditions tested in this experiment (pH, soluble solids content, temperature and time combinations), the *D*-values predicted with that model were considerably greater (ranging from 8 to 28 minutes) than the values obtained experimentally in this study (Table 4). These differences can be attributed to variations within the tested strains and liquid substrates but more likely to variations in the methodology used to determine the thermal resistance parameters. Therefore, as Gabriel (*16*) stated and based on the findings of this study, his model can be safely used to establish a thermal process schedule for acid and acidified liquid products. Unfortunately, due to the apparent overestimation observed in the model, the predictive conditions might not be optimal for practical applications, due to a potential detrimental effect of those processing conditions on organoleptic and nutritional qualities of beverages, and due to increments in production costs by increased processing time and energy consumption.

For apple-carrot juice blends with pH values under 4.5, our results confirmed that the treatment recommended by Mazzota (*27*) and required by the U.S. Food and

Drug Administration of 3 s at 71.1°C is suitable for achieving a greater-than-5-log reductions of the acid-adapted *E. coli* tested in this study. Therefore, the time and temperature combination suggested by Mazzota (*27*) would guarantee the safe thermal processing of this fruit juice blend, regardless of the organic acid used for pH adjustment. Considering the results reported in our investigation and specifically the *D* and *z*-values obtained at a pH of 3.7 for acid-adapted *E. coli* (the combination that experimentally lead to the greater heat tolerance of this pathogen in acidified samples), an example of a treatment that would reduce a population of vegetative *E. coli* O157:H7 C7927 by 5 logs is represented by 2 s at 71.1°C (*z*-value of 5.4°C). These processing conditions are based on the extrapolation of results obtained between 54 and 58°C, and therefore, juice processors and process authorities may want to generate specific data to validate the effectiveness of the suggested treatment.

Furthermore, considering the *D*-values determined at the conditions that triggered the greatest heat tolerance (non-acid-adapted *E. coli*, pH of 4.5 and heating temperature of 54°C), a minimum treatment of 10 s at 71.1°C (*z*-value of 6.2°C) would be required to achieve a 5-log reduction of the tested strain. This agrees with results obtained by Mazzota (*27*), who states that the heat tolerance of pathogens likely to occur in juices including *Salmonella, Listeria monocytogenes* and *E. coli* O157:H7, increases considerably above pH 4. Thus, for the apple-carrot juice blend and similar liquid food products with a pH of 4.5 and above, process authorities and juice processors may want to generate

specific thermal inactivation data related to the most resistant pathogen likely to occur in the product and to optimize the thermal process.

Data from our experiments confirmed that pH, heating temperature, type of organic acidulant and acid adaptation dramatically influence the thermal tolerance response of the *E. coli* O157:H7 C7927 strain studied. Therefore, for the establishment of critical limits for the safe thermal processing of pH controlled juices and similar food products, the process authorities or regulatory agencies must give serious consideration to these parameters.

These results may help processors and process authorities establish the critical limits for safe thermal processing of juices and similar pH controlled foods. The authors also believe that this work will be useful to meet part of the new FDA Food Safety and Modernization Act (FSMA) science-based rules to ensure the safety of acidified and acid foods (*36*).

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CHAPTER 3

EFFECT OF ACID ADAPTATION AND ACID SHOCK ON THERMAL TOLERANCE AND SURVIVAL OF *ESCHERICHIA COLI* O157:H7 AND O111 IN APPLE JUICE[1]

ABSTRACT

Gradual exposure to moderate acidic environments may enhance the thermal tolerance and survival of *Escherichia coli* O157:H7 in acid and acidified foods. Limited studies comparing methodologies to induce this phenomenon have been performed. The effects of strain and physiological state on thermal tolerance and survival of *E. coli* in apple juice were studied. The decimal reduction time (*D*-value) at 56ºC [$D_{56ºC}$] was determined for *E. coli* O157:H7 strains C7927 and ATCC 43895, and *E. coli* O111 at four physiological states: unadapted, acid-shocked (two methodologies used), and acid-adapted cells. The effect of acidulant was also evaluated by determining the $D_{56ºC}$ for the O157:H7 strains subjected to acid shock during 18 h in Trypticase soy broth (TSB) with pH 5 adjusted with hydrochloric, lactic, and malic acids. Survival of the three strains at four physiological states was determined at 1 ± 1ºC and 24 ± 2ºC. Experiments were performed in triplicate. For thermal inactivation, a significant interaction was found between strain and physiological state ($P < 0.0001$). Highest thermal tolerance was observed for the 43895 strain subjected to acid shock during 18 h in TSB acidified with HCl ($D_{56ºC}$ of 3.0 ± 0.1 min), and the lowest for the acid-shocked C7927 strain treated for 4 h in TSB acidified with HCl ($D_{56ºC}$ of

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0.45 ± 0.06 min). Acidulants did not alter the heat tolerance of strain C7927 ($D_{56°C}$ of 1.9 ± 0.1 min) (P > 0.05), but significantly affected strain 43895 (P < 0.05), showing the greatest tolerance when malic acid was used ($D_{56°C}$ of 3.7 ± 0.3 min). A significant interaction between strain, storage temperature, and physiological state was noted during the survival experiments (P < 0.05). *E. coli* O111 was the most resistant strain, surviving 6 and 23 days at 24 and 1ºC, respectively. Our findings may assist in designing challenge studies for juices and other pH-controlled products, where Shiga toxin-producing *E. coli* represents the pathogen of concern.

INTRODUCTION

Apple juice and cider contaminated with *Escherichia coli* O157:H7 and *E. coli* O111 have been the cause of numerous foodborne outbreaks (*7*). Therefore, these pathogens represent a safety concern for these, and other acidic and acidified food products. Moreover, it has been demonstrated that a gradual exposure of certain pathogens (including Shiga toxin-producing *E. coli*) to moderate acidic environments, may enhance the thermal tolerance and survival ability of these microorganisms when present in pH-controlled products (*11, 14*, *16*, *19, 20, 21*). Different methodologies to induce acid-enhanced responses, including acid shock and acid adaptation protocols, have been developed and applied in several microbial challenge studies. Nevertheless, limited studies have been performed to compare these methodologies and their potential differentiated effects on the thermal tolerance and survival of different *E. coli*

strains, which have shown significant variations even within a single serotype (*5*, *9*, *11, 15, 23*). Thus, the lack of comparative studies using different strains and procedures complicates the selection of the most appropriate methodology to conduct safety validations in acidic juices and similar food products.

Prior to this experiment, Ryu and Beuchat (*20*) performed a comprehensive study to determine the survival and growth characteristics of acid-adapted, acid-shocked, and unadapted cells of *E. coli* O157:H7 strain E0139 (venison jerky isolate), inoculated into Trypticase soy broth (TSB) acidified with lactic and acetic acids, and for three commercial brands of apple cider and orange juice. Acid-adapted cells were reported more tolerant when compared to acid-shocked and control cells, and the pathogen survived up to 42 days at 5 and 25ºC in both juices. In this study, only one strain was evaluated. Hsin-Yi and Chou (*11*) studied two *E. coli* O157:H7 strains (ATCC 43889 and ATCC 43895) subjected to acid shock, but acidifying the TSB with HCl, and exposing the culture to the acidified media for 4 h instead of the 2 h indicated by Ryu and Beuchat (*20*). In this case, commercial mango juice, asparagus juice, Yakult, and low fat yogurt were inoculated with acid-shocked and control cells, and survival was determined at 25 and 7ºC. In this study, the 43895 strain survived longer than the 43889 strain in all products, and regardless of the storage temperature and physiological state, the acid shock treatment and low storage temperature increased the survival of both strains.

For the purpose of this study, and as previously stated by Ryu et al. (*21*), the acid shock term was used for cells that have been exposed to an abrupt shift from high to low pH, whereas acid-adapted cells were defined as those that have been exposed to a gradual decrease in environmental pH. Acid adaptation is considered a pre-treatment that more closely mimics what might take place in fermented products in which acids are produced by naturally occurring microbiota or added bacteria (*8*). However, an acid shock scenario is more likely to occur in processing facilities where acidic products and organic acids are commonly used with preservation purposes and to achieve desirable sensory qualities.

The objective of this study was to evaluate the combined effects of strain, physiological state, and acidulant on the thermal tolerance and survival of different pathogenic *E. coli* strains in apple juice. This product was chosen because it is a highly consumed acidic product, and it has been reported that Shiga toxin-producing *E. coli* has survived in this product and caused hemorrhagic colitis outbreaks (*7*). This research aims to help juice processors and process authorities to establish the most appropriate experimental conditions for the execution of microbial challenge studies on pH-controlled juices and similar liquid food products.

MATERIALS AND METHODS

Apple juice. Shelf-stable, preservative-free and single-strength 100% apple juice was purchased at a local grocery store (Geneva, NY), and kept frozen at –23ºC

until used. After thawing, 9 ml of apple juice were dispensed aseptically in a sterile test tube, pasteurized at 82ºC for 6 minutes, and immediately cooled. The pasteurization step was performed to avoid the presence of any potential unwanted microbiota that would interfere with the *E. coli* quantification during the heat tolerance and survival determinations. To verify the absence of background microbiota, three replicate samples of pasteurized apple juice were plated on plate count agar (PCA), and acidified (pH 3.5) potato-dextrose agar (PDA) (Difco, BD, Sparks, MD), and incubated at 35 ± 2ºC for 24 and 48 h, respectively, in order to determine total plate, as well as molds and yeasts counts.

Physicochemical measurements. The juice's pH was measured with a Thermo Scientific Orion 2 Star pH meter (Thermo Fisher Scientific, Beverly, MA). The soluble solids content were determined using a Leica Auto Abbe refractometer model 10500-802 (Leica Inc., Buffalo, NY). Total titratable acidity was measured with a G20 compact titrator (Mettler Toledo, Schwerzenbach, Switzerland), and turbidity was determined using a HACH 2100P portable turbidimeter (Hach Company, Loveland, CO). All physicochemical analyses were performed in triplicate.

Bacterial strains and culture conditions. A single isolated colony of three Shiga toxin-producing *E. coli* strains, *E. coli* O157:H7 (strains C7927 and ATCC 43895), and *E. coli* O111 (strain 04-11953), obtained from the Food Microbiology Laboratory at the New York State Agricultural Experiment Station (Geneva, NY)

was transferred into 10 ml of TSB (Difco, BD, Sparks, MD), and incubated for 20 ± 2 h at 35 ± 2ºC (to stationary-phase) in an Innova 2300 rotatory platform shaker (New Brunswick Scientific Co., Edison, NJ) at 250 rpm. These cultures were used as the unadapted (control) strains and then subjected to the acid adaptation and acid shock protocols described below. The *E. coli* O111 and *E. coli* O157:H7 C7927 strains correspond to clinical isolates, whereas the *E. coli* O157:H7 strain 43895 corresponds to a food isolate. The C7927 strain was originally isolated from a patient who had consumed contaminated apple cider that caused an outbreak (*24*), and the 43895 strain was originally isolated from raw hamburger meat, also implicated in a hemorrhagic colitis outbreak (*26*).

Acid adaptation and acid shock induction. A total of four methods were used for preparing cells at different physiological states including unadapted-control (described above), acid-adapted, and acid-shocked (two methodologies used). Acid adaptation was produced according to the protocol followed by Breidt et al. (*3*), where one isolated colony of each strain was inoculated separately into 5 ml of TSB supplemented with 1% glucose, purchased from Fisher Scientific (Fair Lawn, NJ). Cultures were grown statically at 35 ± 2ºC for 16 ± 2 h to induce the enhanced acid tolerance response by a gradual decrease of pH to an average value of 4.71 ± 0.05. After incubation, cells were concentrated by centrifugation at 2000 *x g* and 10ºC for 10 minutes using an Eppendorf 5417R microcentrifuge (Hamburg, Germany), and resuspended in 1 ml of Butterfield's buffer phosphate diluent (BPD). As a modification to the original protocol (*3*), the Luria-Bertani

broth was substituted by TSB and the sterile saline solution (0.85% NaCl) was replaced with BPD. Both changes were applied to prevent the inclusion of an additional source of cell stress due to the presence of high concentrations of sodium chloride, a compound that is not naturally found (at high concentrations) in apple juice and other fruit juices.

The acid shock effect was induced on the three selected strains following two different methodologies. For the first protocol, referred as AS1, as stated by Enache et al. (*9*), a loopful of unadapted-control cells of *E. coli* (obtained as indicated in Bacterial Strains and Culture Conditions above) was transferred into 5 ml of TSB with a pH of 5, adjusted by the addition of HCl 1 N (Fisher Scientific, Fair Lawn, NJ), and incubated overnight (18 ± 2 h) at 35 ± 2ºC. After incubation, 1 ml of the acid-shocked culture was centrifuged at 8800 *x g* for 4 minutes in an Eppendorf 5415C microcentrifuge (Hamburg, Germany), and the pellet was resuspended in refrigerated 0.1 M citrate buffer (pH 4), and stored at 4 ± 1ºC for 18 h before use. The acid-shocked cells were also obtained following the protocol reported by Hsin-Yi and Chou (*11*), and referred as AS2, in which a volume of 1 ml of unadapted-control cells of *E. coli* was centrifuged at 8800 *x g* for 12 minutes in an Eppendorf 5415C microcentrifuge. The cell pellets were washed with BPD and resuspended in 1 ml of TSB with a pH adjusted to 5 by adding HCl 6 N. Cultures were statically incubated at 35 ± 2ºC for 4 h before use.

Thermal tolerance determination. The decimal reduction times (*D*-values) were determined according to the methodology described by Usaga et al. (*25*), where 9 ml of apple juice were inoculated with 1 ml of each culture, resulting in an initial population of 10^7 to 10^8 CFU·ml^{-1}. A volume of 20 µl of inoculated apple juice was injected into five glass melting point capillary tubes (1.5 to 1.8 by 100 mm; Kimble Chase, Vineland, NJ) using a 1 ml syringe and a repeater dispenser (Hamilton Co., Reno, NV). After flame sealing, capillary tubes were heat treated in water test tubes contained in a stirred water bath set at a temperature of 56ºC. The selected sampling times differed depending on the strain and physiological state, but all the thermal death time curves where characterized by at least 5 sampling points, 4-log reductions, and a coefficient of determination (r^2) greater than 0.9. Juice samples that represented the time zero in the thermal death curves were considered the nonheated controls. After heating, capillary tubes were cooled in an ice bath and, with the objective of decontaminating their exterior and ending the thermal treatment, the tubes where immediately placed in test tubes containing 70% cold ethanol contained in an ice water bath. The excess of ethanol was removed by blotting the capillaries. The five capillary tubes were crushed with a sterile glass rod in a milk dilution bottle containing 20 ml of 0.1% sterile peptone water. A minimum detection limit of 10^2 CFU·ml^{-1} was thus obtained. Appropriate serial dilutions in sterile 0.1% peptone water were aseptically plated by duplicate in petri dishes where 20 ml of Trypticase soy agar (TSA) (Difco, BD, Sparks, MD) was pour-plated. Petri dishes were incubated for 20 ± 2 h at 35 ± 2ºC before counting colonies. The *D*-values were

calculated as the inverse negative slope of the linear regression line obtained from plotting the log number of survivors against the sampling times.

Effect of acid adaptation and acid shock on the thermal tolerance of *E. coli.* We evaluated the effect of the four physiological states on the *D*-values for each of the three enterohemorrhagic *E. coli* strains. A total of three independent biological replicates were performed of each treatment (physiological state × strain).

Effect of acidulants on the thermal tolerance of acid-shocked *E. coli.* We studied the effect of the acidulant used in the acid shock protocol (AS1) on the *D*-values for the two *E.coli* O157:H7 strains. Three organic acids 85% malic (6 M), 85% lactic (9 M) and 85% acetic (14 M) were used instead of HCl (1 M). Acids were purchased from Fisher Scientific (Fair Lawn, NJ). A total of three independent biological replicates were performed of each treatment (acidulant × strain).

Survival of *E. coli* in apple juice. We evaluated the effect of the four physiological states on the survival for each of the three enterohemorrhagic *E. coli* strains, at two storage temperatures: refrigeration (1 ± 1ºC), and room temperature (24 ± 2ºC). A volume of 9 ml of pasteurized apple juice was inoculated with 1 ml of the three tested *E. coli* strains at the four physiological states. Initial *E. coli* counts were determined on day 0 by using the spread plate

techniques on TSA, and sampling intervals for subsequent counts depended on the total bacterial population at each sampling time (spread or pour plate techniques where used accordingly). Petri dishes were incubated for 20 ± 2 h at 35 ± 2ºC before enumeration. A total of three independent biological replicates were performed for each treatment (physiological state × strain × storage temperature).

Statistical Analysis. Two- and three-way analyses of variance (ANOVA), Tukey's honestly significant difference (HSD) and Student's t tests for means comparisons were performed using JMP® version 11 (SAS Institute Inc., Cary, NC). Three values per treatment (from three independent replicates) were used in each case. Differences were considered significant at a P value of < 0.05.

RESULTS AND DISCUSSION

Apple juice was characterized by a pH of 3.627 ± 0.006, 11.873 ± 0.006 ºBrix, a total titratable acidity of 0.35 ± 0.01 % (grams of malic acid per 100 grams of juice) which is equivalent to an average of 0.028 M of malic acid, and a turbidity of 1.35 ± 0.01 nephelometric turbidity units (NTU), representing a clear juice. The absence of background microbiota was confirmed by negative results on the total plate count and molds and yeasts count.

Effect of acid adaptation and acid shock on the thermal tolerance of *E. coli.* In order to determine whether the tested methods had a significant effect on the thermal tolerance of the *E. coli* strains, a full factorial design with three levels for strain (*E. coli* O157:H7 C7927 and ATCC 43895, as well as *E. coli* O111), and four levels for physiological state (unadapted-control, AS1, AS2, and acid-adapted cells) was built, with the measured *D*-values at 56ºC as the response. Representative thermal death time curves for *E. coli* O157:H7 strain C7927 are depicted in Figure 2. These curves demonstrate that there were no deviations from the linear decline in the log number of survivors over time. Similar curves were obtained for all experimental conditions and used to calculate all the *D*-values reported on this study. A significant interaction between strain and physiological state ($P < 0.0001$) was observed after performing a two-way ANOVA, and the resulting model showed a coefficient of determination (r^2) of 0.95. *Post hoc* multiple comparisons were performed using Student's t test with a Bonferroni correction, which allowed to compare mean values and determine significant

differences in physiological states within strains and in strains within physiological states.

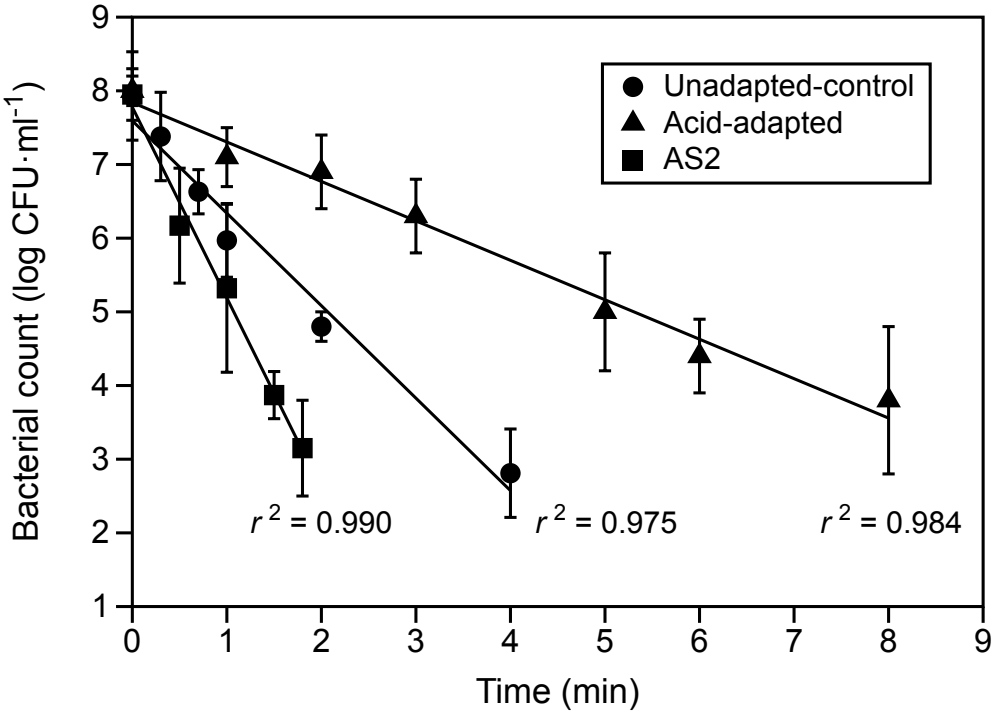
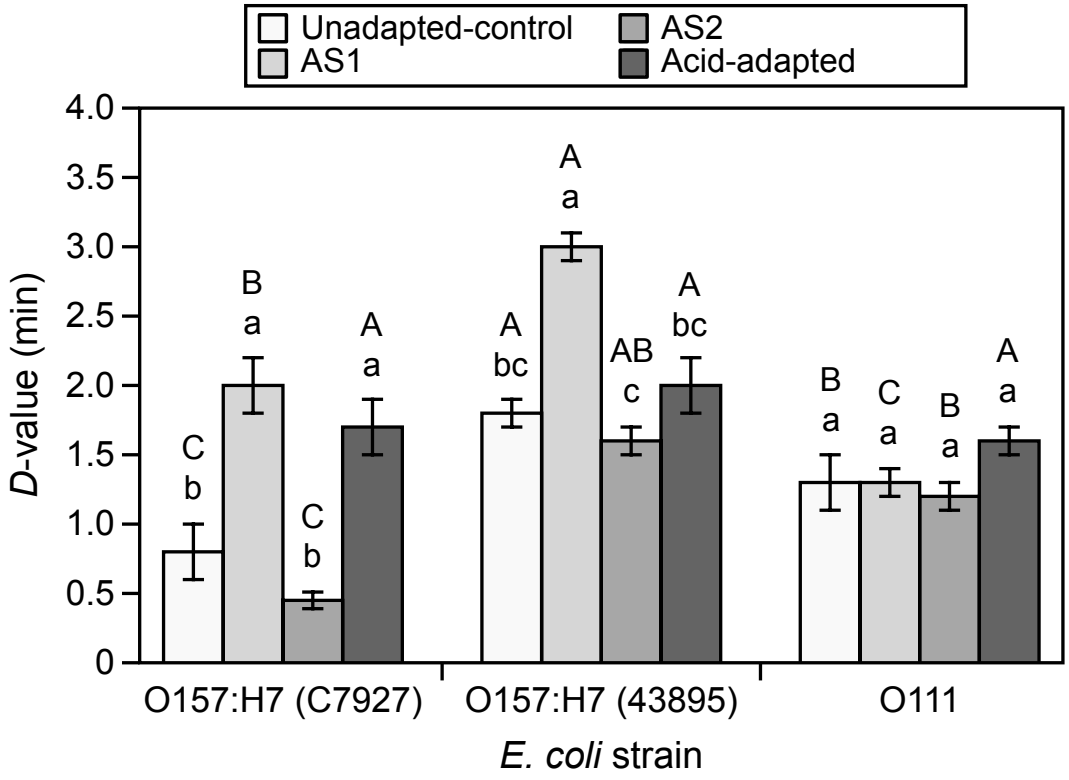


Figure 2. Representative thermal death time curves determined in apple juice (pH 3.6) at 56ºC for unadapted-control, acid-adapted, and acid-shocked (AS2) *E. coli* O157:H7 strain C7927. Error bars represent standard deviations (n = 3).

As depicted in Figure 3, the greatest thermal tolerance was observed for acid-shocked *E. coli* O157:H7 ATCC 43895 subjected to AS1 (3.0 ± 0.1 min), whereas the lowest was obtained for *E. coli* O157:H7 strain C7927 subjected to AS2 (0.45 ± 0.06 min). For the two O157:H7 strains tested, the thermal tolerance of the bacteria was found significantly higher when subjected to AS1, as compared with the unadapted-control counterparts. Moreover, the *D*-values for these strains were also consistently higher than those obtained for acid-adapted cells (Figure 3). Worth noting, the thermal tolerance of these O157:H7 strains was greatly influenced by the time of exposure of the culture to the

acidic growing media (18 h in AS1 versus 4 h in AS2, which translated to lower *D*-values in AS2 than in AS1), indicating that this time represents a critical factor that influences the enhanced thermal tolerance response on the pathogenic *E. coli* strains tested in this study. In a previous experiment conducted in our laboratory (*25*), we also found that the *D*-values at 54, 56 and 58ºC for *E. coli* O157:H7 C7927 (one of the strains evaluated on this study), determined in an apple-carrot juice blend acidified with different acidulants and at different pH values, increased significantly due to the acid shock treatment (AS1).



[a] *D*-values not sharing a common lowercase letter represent significantly different values ($P < 0.05$) when comparing physiological states within strains. D-values not sharing a common uppercase letter represent significantly different values ($P < 0.05$) when comparing strains within physiological states. Post hoc multiple comparisons were performed using Student's t test with a Bonferroni correction, following a two-way ANOVA.

Figure 3. *D*-values of three *E. coli* strains at four physiological states, determined at 56ºC in apple juice (pH 3.6). Error bars represent standard deviations (n = 3).[a]

It has been previously reported that during the acid shock many physiological changes (responsible of an enhanced acid tolerance response) such as the production of protective acid stress proteins that in *E. coli* are regulated by σ^s (*rpoS*), are occurring (*6*). However, it is possible that during the application of AS2 these changes are not completely developed and therefore, a cell damaging effect due to the abrupt exposure to a low pH, is exceeding the expected protective effect induced by the acid shock treatment. Consequently, it may be causing the adversely affected heat tolerance. Previously, Ryu and Beuchat (*20*) found that the heat tolerance of *E. coli* O157:H7 (strain E0139) in apple cider can be substantially enhanced by acid adaptation compared to acid shock. In that case, acid-shocked cells were obtained by adjusting a 16 h *E. coli* culture in TSB with a pH of 4.8 by adding lactic acid, and then incubating for 2 h at 37ºC. Differences in the conclusions from this and our study may result from different times of acid exposure during the acid shock, differences on the acidulants used to adjust the pH of the growing media, and natural and intrinsic variations among the strains. Worth noting, and as indicated in the methodology section above, the application of the AS1 treatment in contrast with AS2 includes additional cold and acid shock treatments for 18 h at 4ºC on a citrate buffer (pH 4, 0.1 M) that may also influence the *E. coli* thermal tolerance observed in this study. These differences in the methods, besides variables such as the methodologies used to experimentally determine the *D*-values, and the acid concentration and ionic strength might also explain the observed disparities.

For the case of *E. coli* O111, in agreement with Ryu and Beuchat (*20*), the highest *D*-value (1.6 ± 0.1 min) was observed when the culture was subjected to acid adaptation instead of acid shock, and the time of acid shock did not significantly affect the heat tolerance of this strain (comparing AS1 to AS2) ($P > 0.05$). Interestingly, when acid adaptation was applied, no significant differences in the measured *D*-values was observed between the three enterohemorrhagic strains (Figure 2). However, when the *D*-values were obtained using unadapted-control, and acid-shocked cultures were compared between strains, a significant effect ($P < 0.05$) was observed. Buchanan and Edelson (*4*) evaluated the acid tolerance of seven enterohemorrhagic *E. coli* strains (*E. coli* O157:H7 strains B1409, 45753-35, 30-2C4, 932, Ent-C9490, A9124-C1, and *E. coli* O111:H^- strain 95JB1) subjected to acid adaptation. All these strains were found to be acid-tolerant, but in agreement with our results, and regardless of the different strains evaluated, the authors reported significant differences on the acid tolerances among the strains, and consequently, they classified the isolates in three categories depending on the acquired acid-tolerance when pre-adapted. Buchanan and Edelson (*4*) explained the variations among the strains indicating that pH-independent and pH-dependent stationary-phase acid tolerance phenotypes may exist among enterohemorrhagic *E. coli* strains, and postulated that the isolates selected on their study may represent three genotypically distinct groups or a spectrum of strains with different *rpoS* expression or different levels of production of protective cellular components (*4*). These characteristics may also explain the dissimilar thermal tolerance responses reported in our study. Moreover, the authors (*4*) also suggested that the strains that showed enhanced acid tolerance responses when acid-adapted, like the

case of the three strains evaluated in our study, appear to resemble a pH-dependent stationary-phase acid tolerance response previously reported for *Salmonella* Typhimurium (*4, 10*, *13*), whereas the strains that did not show an increased tolerance could have a pH-independent acid resistance response instead.

Table 5. Initial counts in apple juice samples (pH 3.6) inoculated with *E. coli* O157:H7 strains C7927 and 43895, subjected to acid shock for (18 ± 2 h) in TSB acidified to pH 5 (AS1) by adding four different acids.[a]

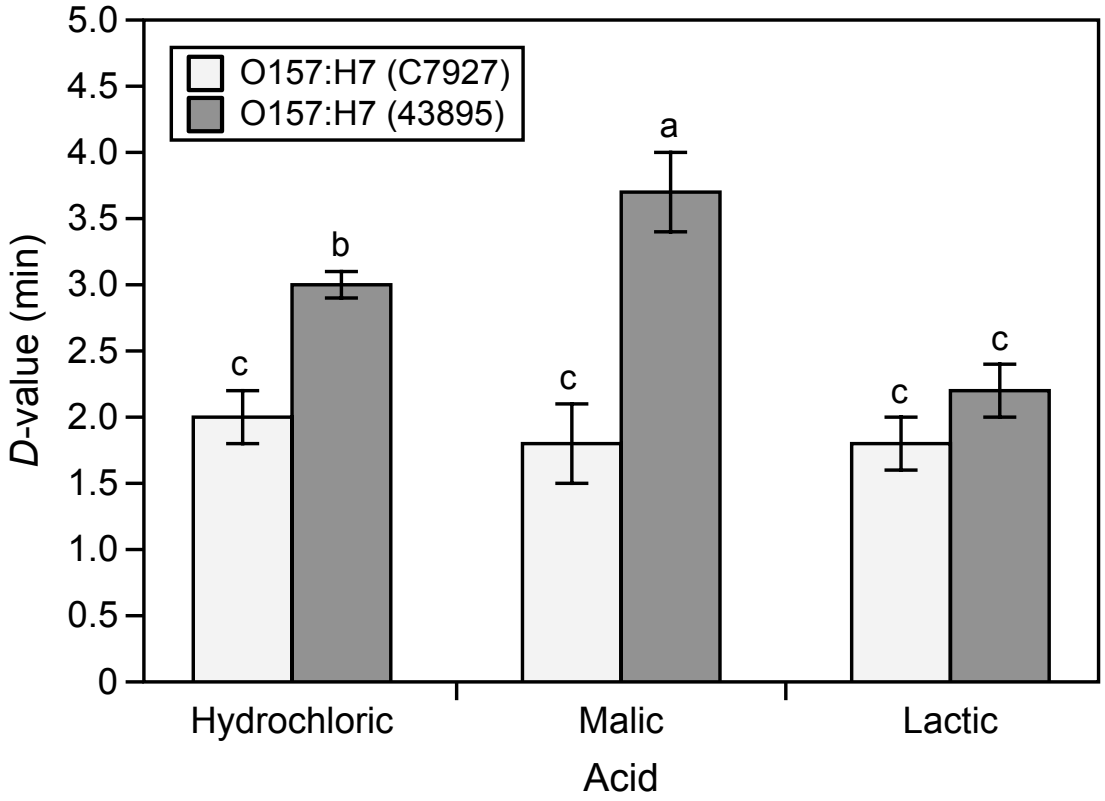
Acid	*E.coli* O157:H7 strain	
	43895	C7927
Hydrochloric	7.9 ± 0.6	7.0 ± 0.2
Malic	7.9 ± 0.1	6.0 ± 0.7
Lactic	7.4 ± 0.5	7.2 ± 0.2
Acetic	5.5 ± 0.2	5.59 ± 0.06

[a] Values are the average ± standard deviation (n = 3).

Effect of acidulants on the thermal tolerance of acid-shocked *E. coli.* Since the greatest thermal tolerance response (highest *D*-values) was exhibited by the two acid-shocked *E.coli* O157:H7 strains subjected to AS1, the effect of inducing this phenomenon by acidifying the growing media with different organic acids (malic, lactic and acetic) instead of HCl was studied. The *E. coli* counts of the nonheated controls are indicated in Table 5. As observed, when the three strains where exposed to the acid shock in media containing acetic acid, the population of *E. coli* was lower when compared to counts of populations grown when other organic and inorganic acids where used. This result agrees with Ryu et al. (*21*), who found that acetic acid was the most lethal acidulant for *E. coli* O157:H7 (strain E0139), followed by lactic and malic acids,

when tested over a pH range from 3.9 to 5.4 and at 37ºC. Similarly, Deng et al. (*8*) reported that three strains of *E. coli* O157:H7 (30-2C4, ENT-C9490, and SEA-13B88) were markedly less tolerant to acetic acid than citric and malic acids when 18 h stationary-phase cultures were surface-plated onto TSB acidified at six different pH values (5.4, 5.1, 4.8, 4.5, 4.2, and 3.9), and incubated at 37ºC for at least 48 h. It is important to note that comparisons of the antimicrobial effect of different organic acids on Shiga toxin-producing *E. coli* should consider the relative effectiveness of the acid. Therefore, comparisons at different pH values should be based on the concentration of the effective form of the acids, information that in some publications has not been provided or does not match with the conditions evaluated in the present study. Considering the low initial counts obtained in the presence of acetic acid (Table 5), and that the detection limit associated to the thermal inactivation methodology is 10^2 CFU·ml^{-1}, it was deemed not feasible to perform the *D*-values determination on these samples and still obtain a thermal death curve extended for at least 4-log reductions, condition that was pre-established by the authors as a requirement to calculate the *D*-values. Given this situation, and as a preliminary attempt to determine the *D*-value of acid shocked *E. coli* in TSB acidified with acetic acid for one of the O157:H7 strains (C7927), a higher concentration of initial inoculum (1 ml instead of a loopfull) was subjected to AS1 using TSB acidified with this acid, and the $D_{56ºC}$ was determined. However, the resulting *D*-value (1.1 ± 0.1 min) was not significantly different ($P > 0.05$) than the value observed for the unadapted-control counterpart (0.8 ± 0.2 min) and it was significantly lower ($P < 0.05$) than the values reported when the other acidulants were used (Figure 4). This result suggests that AS1 does not

enhance the acid tolerance of this strain when acetic acid is used, and possibly, that this organic acid is causing a damaging cell effect that negatively affects the thermal tolerance of the bacteria. Thus, for the acidulants that allowed the determination of the *D*-values, complying with all our pre-established requirements, a complete factorial design was used with two levels for strain (C7927 and 43895), and three levels for acidulant (hydrochloric, malic, and lactic acids). Results are summarized in Figure 4.



[a] *D*-values not sharing a common lowercase letter represent significantly different values ($P < 0.05$) based on *post hoc* multiple comparisons with a Tukey correction following a two-way ANOVA.

Figure 4. *D*-values determined at 56ºC in apple juice (pH 3.6) of two *E. coli* O157:H7 strains subjected to acid shock (AS1) in TSB with a pH of 5, adjusted by the addition of hydrochloric, malic, and lactic acid. Error bars represent standard deviations (n = 3).[a]

A significant interaction (P = 0.0002) between strain and acidulant was observed after performing a two-way ANOVA, and the resulting model showed a coefficient of determination (r^2) of 0.93. The acidulants did not alter the heat tolerance of the C7927 strain (P > 0.05) but significantly affected the 43895 strain (P < 0.05), which showed the greatest tolerance when malic acid was used (Figure 4). Based on these results, and considering that organic rather than inorganic acids are often used by the food industry, and as previously recommended by Deng et al. (*8*), it seems that for studying the acid tolerance of Shiga toxin-producing *E. coli* strains in acidic and acidified products, it would be preferable to acidify the media by adding an organic acid with a lower strength such as malic acid (pK_{a1} 3.40 and pK_{a2} 5.20), instead of an inorganic acid such as hydrochloric acid or an organic acid with a higher pK_a, for example lactic acid (pK_{a1} 3.86) and acetic acid (pK_a 4.79), rather than malic acid.

Survival of *E. coli* in apple juice. The survival curves for the three Shiga toxin-producing *E. coli* strains determined at room temperature and under refrigeration are shown in Figure 5 and 6, respectively. A full factorial design was used to evaluate the effect of strain, storage temperature, and physiological state on the survival of these pathogenic strains in the apple juice. The experimental design included three levels for strain: *E. coli* O157:H7 C7927 and ATCC 43895, as well as *E. coli* O111; two levels for storage temperature: 24 ± 2ºC and 1 ± 1ºC; and four levels for physiological state: unadapted-control, acid-shocked (induced by AS1 and AS2), and acid-adapted. The survival response corresponds to the day when the *E. coli* count on each of the three independent replicates and at each of the tested conditions, was zero. The three-way

ANOVA showed a significant triple interaction between strain, storage temperature, and physiological state (P = 0.04). Therefore, the data were further analyzed by performing two independent two-way ANOVAs for each temperature, each analysis with two factors: strain and physiological state. The two resulting models showed coefficients of determination (r^2) of 0.89 for samples stored at room temperature, and 0.84 for the case of refrigerated samples.

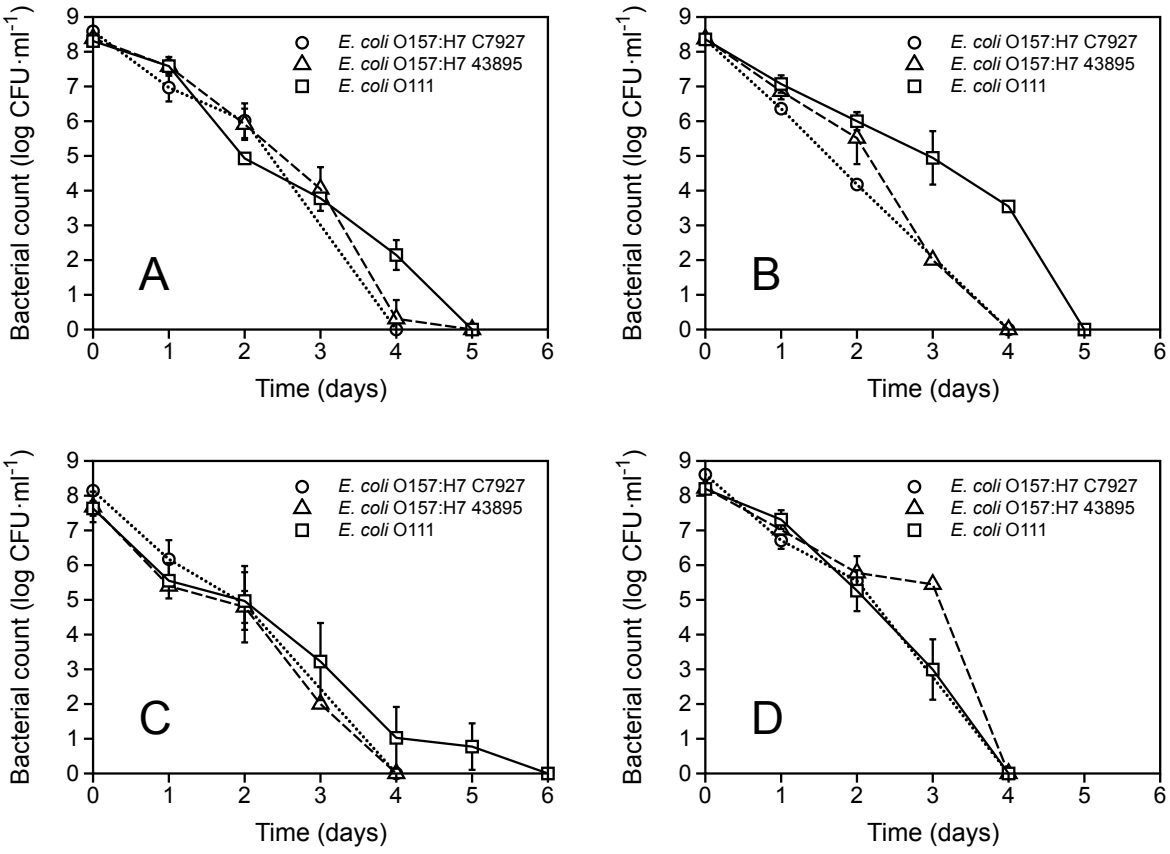


Figure 5. Survival curves for three *E. coli* strains at four physiological states: (A) unadapted-control, (B) acid-adapted, (C) acid-shocked (AS1), and (D) acid-shocked (AS2), determined in apple juice (pH 3.6) stored at room temperature (24 ± 2ºC). Error bars represent standard deviations (n = 3).

In the case of samples stored at 24 ± 2ºC (Figure 5), a significant interaction between strain and physiological state was found ($P < 0.0001$). Furthermore, the acid-shocked *E. coli* O111 strain subjected to AS1 (Figure 5C) was the most resistant strain, and survived for up to 6 days.

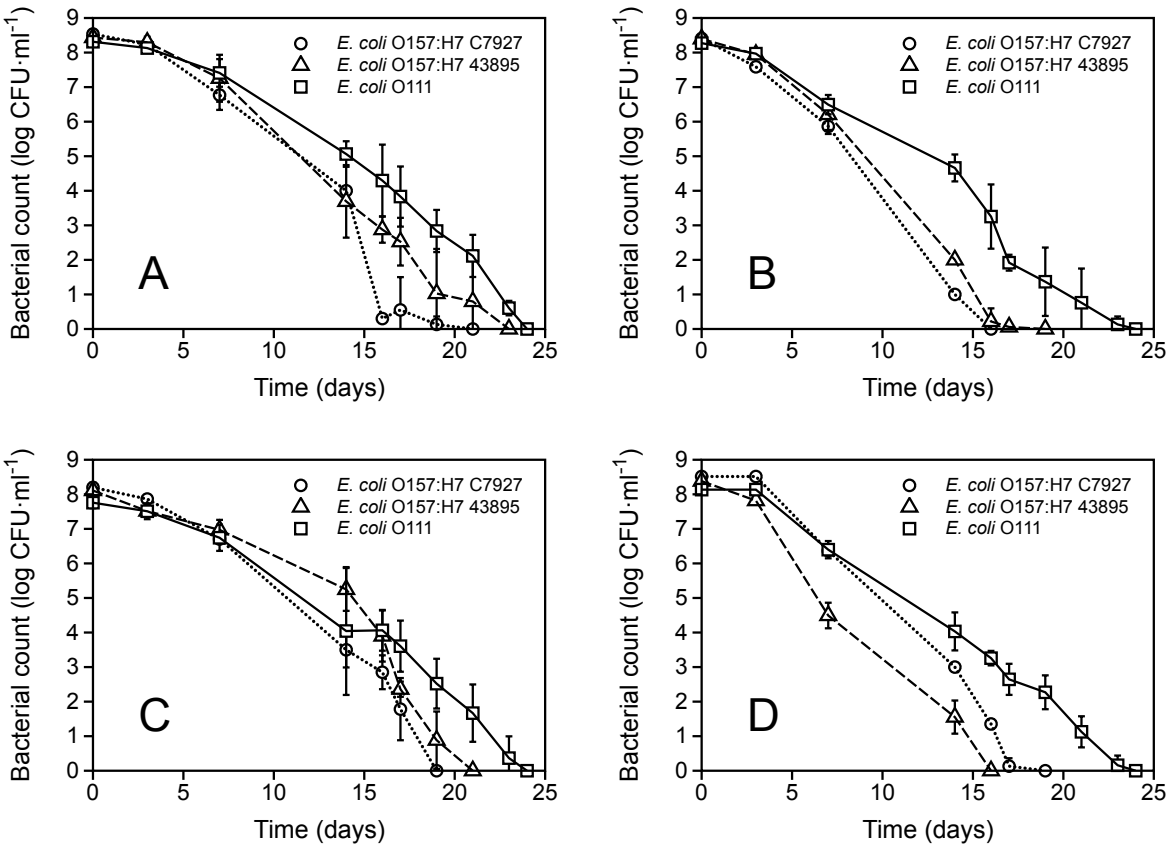


Figure 6. Survival curves for three *E. coli* strains at four physiological states: (A) unadapted-control, (B) acid-adapted, (C) acid-shocked (AS1), and (D) acid-shocked (AS2), determined in apple juice (pH 3.6) stored under refrigeration (1 ± 1ºC). Error bars represent standard deviations (n = 3).

For refrigerated samples (Figure 6), the interaction between strain and physiological state was deemed nonsignificant ($P = 0.14$). Thus, after removing the two-way interaction term, results showed that both strain and physiological state had significant

effects on the survival response ($P < 0.05$). Furthermore, after *post hoc* multiple comparisons with a Tukey correction, the *E. coli* O111 was found on average significantly more resistant than the two *E. coli* O157:H7 strains ($P < 0.05$) surviving up to 23 days, and no significant differences between the survival of both O157:H7 strains were noted ($P > 0.05$) (on average, strain 43895 survived for 19 days and C7927 for 18 days). Moreover, no significant differences in the survival response were observed between strains subjected to acid adaptation (Figure 6B) and acid shock by using the protocol AS2 (Figure 6D). On average, the observed survival of the strains subjected to these physiological states was significantly lower when compared with the observed responses in the unadapted-control (Figure 6A) and acid-shocked cultures subjected to AS1 (Figure 6C), which showed the highest survival responses.

Overall, the lowest temperature increased the survival response of the three tested strains, confirming that as suggested by Lin et al. (*12*), and Miller and Caspar (*17*), *E. coli* O157:H7 is acid tolerant and particularly tolerant at low temperatures. Previously, Zhao et al. (*27*) studied the fate of unadapted *E. coli* O157:H7 strain C7927 (one of the strains used on this study), and in agreement with our findings, the authors found a significant effect of storage temperature on the survival of this pathogen in apple cider. A survival of 10 to 31 days at 9ºC, and 2 to 3 days at 25ºC (pH 3.6 to 4.0) was observed. However, in that study the physiological state was not included as a variable in the experiment. In contrast, Ryu and Beuchat (*20*) reported a survival of *E. coli* O157:H7 (E0139) in apple juice of up to 42 days at 5 and 25ºC but they did not find an effect of the storage temperature or physiological state on the bacteria's survival

response. Variations in the survival of Shiga toxin-producing *E. coli* strains are likely due to differences among strains, physicochemical properties of juices (including its pH, soluble solids, suspended insoluble solids, etc.) storage temperatures, as well as the acid shock or acid adaptation methodology used to induce the enhanced tolerance response to acidic environments.

Data from our study suggests that for this particular fruit juice and considering the test strains, *E. coli* O157:H7 ATCC 43895 represents the most heat tolerant strain when subjected to acid shock during 24 h in TSB acidified with malic acid, the most abundant organic acid naturally present in apple juice. The heat treatment suggested by Mazzota (*16*) and recognized by the U.S. Food and Drug Administration for acidic juices is based on a *D*-value of 7 minutes at 56ºC which exceeds the greatest *D*-value obtained in this experiment ($D_{56^{\circ}C}$ of 3.7 ± 0.3 min), thus confirming that the heat treatment currently recommended of 3 s at 71.1ºC (z = 5.3ºC) is suitable to ensure a 5-log reduction of *E. coli* O157:H7 ATCC 43895 in apple juice.

For survival studies, our results indicate that *E. coli* O111 should be used as the strain of reference in apple juice, and that the survival of this pathogen is dramatically enhanced when refrigerated conditions are used. This latter finding stresses the safety concerns associated to acid and acidified products that are kept under refrigeration but have not received a microbial killing step. In addition, we confirm that the variables strain, physiological state, acidulant and time of exposure used to induce the acid shock (main difference between AS1 and AS2 protocols) may dramatically influence the

thermal tolerance and survival responses of Shiga toxin-producing *E. coli*. Hence, for the performance of challenge studies oriented to determine and validate critical limits for safe processing of pH-controlled juices and similar products, process authorities and regulatory agencies must carefully consider these experimental variables and their potential interactions. Therefore, we recommend that the selected microorganism should always represent the most resistant strain for the given product and under its processing conditions. The use of three to five-strain cocktails is a practice that has been widely extended in the design of validation studies involving *E. coli* and other pathogens (*1, 2*, *18, 22*) but for acidic and acidified products, we suggest that these cocktails should forcefully include strains that have shown exceptional acid tolerance. Likewise, it becomes relevant to study the effect of other variables, such as the pH of the media used to induce the acid shock, the application of an additional cold and acid shock treatment such as the applied in the case of AS1, as well as the concentrations of soluble and insoluble solids in the juices, on the heat tolerance and fate of these and other acid-tolerant pathogens.

The results reported in this study aim to facilitate the selection and standardization of the most appropriate conditions required to performed safety validations in apple juice and similar acidic fruit juices. This information may be used as a guideline for determining the proper experimental conditions to conduct microbial challenge studies, even with other food products in which different foodborne pathogens have shown exceptional tolerance to adverse environmental conditions.

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CHAPTER 4

TIME AFTER APPLE PRESSING AND CONCENTRATION OF INSOLUBLE SOLIDS INFLUENCE THE EFFICIENCY OF THE UV TREATMENT OF CLOUDY APPLE JUICE

ABSTRACT

The effects of suspended insoluble solids (SIS) concentration and particle size, and the time after apple pressing on the efficiency of UV treatment of cloudy apple juice were evaluated. Clear model solutions (formulated to resemble physicochemical characteristics of apple juice) and commercial apple juices, containing different solids concentrations, were treated using a *CiderSure* 3500 reactor at 14 $mJ \cdot cm^{-2}$ UV dose. Particle size effect was assessed on model solutions treated at UV dose of 7 $mJ \cdot cm^{-2}$. The juice flow rate through the UV machine was determined. All samples were inoculated with *Escherichia coli* ATCC 25922 (10^6 to 10^7 $CFU \cdot ml^{-1}$) and UV treated at a fixed flow rate of 214.5 $ml \cdot s^{-1}$. Log reductions were calculated. SIS did not significantly affect the juice flow rate ($p > 0.05$) but increasing SIS concentrations adversely affected the inactivation of *E. coli* ($p < 0.05$). For solutions treated at 7 $mJ \cdot cm^{-2}$, a negative linear relationship between SIS and flow rate was observed and particle size significantly affected the flow rate ($p < 0.05$). A negative effect of time after apple pressing on the juice flow rate was observed and this effect was found apple varietal-dependent, thus better UV efficiency will be attained if time after pressing is minimized.

INTRODUCTION

Numerous outbreaks associated with the consumption of contaminated and unpasteurized apple juice around the world (Cody et al. 1999; Danyluk et al. 2012; Steele et al. 1982) have stressed the importance of including a microbial killing step during the processing of these products.

In recent years, due to a global increased consumer demand for more fresh-like beverages with enhanced nutritional properties, the application of ultraviolet (UV) light technology, an affordable nonthermal treatment (Choi & Nielsen 2005; Keyser et al. 2008; Murakami et al. 2006), has attracted the interest of the juice and beverage industries and in particular of small and medium size apple orchards interested in producing clear and cloudy, refrigerated apple juices. Since 2000, the Food and Drug Administration in the United States has recognized UV as an alternative to thermal pasteurization of juices (FDA 2013). This treatment has been proven effective against the pathogens of concern in apple juice including *Escherichia coli* O157:H7 and *Cryptosporidium parvum* (Basaran et al. 2004; Hanes et al. 2002; Oteiza et al. 2005; Quintero-Ramos et al. 2004). Moreover, the technology has shown some advantages in comparison with traditional heat treatments including limited changes in physical, chemical and nutritional properties of juices (Caminiti et al. 2011; Tran & Farid 2004) and degradation of patulin in apple juice (Assatarakul et al. 2011; Dong et al. 2010). Notwithstanding, the application of UV is restricted for certain beverages due to the presence of compounds that increase the UV absorptivity of juices, such as colored compounds and particulate matter, interfere with the UV light's penetration and reduce

the antimicrobial capacity of the technology (Koutchma 2009; Koutchma & Parisi 2004; Oteiza et al. 2005).

Previous studies have demonstrated that suspended insoluble solids (SIS) represent a major concern in UV disinfection of water (Brahmi et al. 2010; Whitbay & Palmateer 1993; Winward et al. 2007). Nevertheless, only a few reports regarding this phenomenon in juices have been published to date. Koutchma et al. (2004) examined the effect of particulate matter on the inactivation of *E. coli* K-12 in cloudy apple juice subjected to 14 $mJ \cdot cm^{-2}$ UV dose and using a *CiderSure* 1500 UV thin film reactor. Increasing the turbidity up to 2400 nephelometric turbidity units (NTU) negatively impacted the effectiveness of the treatment resulting in lower log reductions. Nonetheless, this evaluation was performed using a laminar flow rate, while a turbulent flow regime is a condition required by the FDA, to ensure the safety of UV treated juices (FDA 2013). Murakami et al. (2006) evaluated the same effect in a model apple juice solution with turbidities between 0.5 and 858 NTU and treated with UV doses ranging from 2 to 36 $mJ \cdot cm^{-2}$. A significant effect of SIS on the UV inactivation of *E. coli* K-12 was also observed and explained by the seeming layering of light penetration, which causes the portion of the product closest to the UV-light source to be sterilized effectively, while the farther portion may not be equally treated. However, the effect of turbulence was not evaluated and the extrapolation of the results for juices with higher solids content has some limitations.

Similarly, very little information has been published regarding the change in color of apple juice (browning) due to enzymatic reactions on the efficiency of UV light treatments. Koutchma et al. (2004) studied this effect adding a colored compound to a model solution and found that increasing the absorbance of the solution resulted in lower inactivation levels of *E. coli* K-12. However, results were not related to the actual time after apple pressing and corresponding color change during apple juice manufacturing, and its impact on the productivity of the UV system was not assessed.

This study aims to address some of the existing gaps in the literature regarding the influence of the presence of insoluble solids and the darkening of apple juice after apple pressing on the effectiveness of the UV treatment of cloudy apple juice when using a commercial-scale processing reactor. The elucidation of this information will contribute to understand how these variables may affect the inactivation of *E. coli* and the productivity of UV light systems when treating apple juice and similar liquid products.

MATERIALS AND METHODS

UV processing unit

UV treatments were carried out at a wavelength of 254 nm and at UV doses of 14 $mJ \cdot cm^{-2}$ or 7 $mJ \cdot cm^{-2}$ accordingly. Treatments were performed using a *CiderSure* 3500 commercial UV juice-processing unit (FPE Inc., Rochester, USA), which comprises a stainless steel housing and an inner quartz tube. Beverages are pumped through the system using a positive displacement pump and exposed to eight or four low-pressure mercury lamps depending on the selected UV dose (Quintero-Ramos et

al. 2004). This machine has two settings: “fixed UV dose” and “fixed flow rate” and is equipped with two UV light sensors (at the bottom and top of the outer cylinder) that measure the UV transmittance through the juice every 50 ms. Based on those measurements, when the apparatus is working under the “fixed UV dose” mode, it has been programmed to automatically adjust the pump flow rate ensuring a constant UV dose exposure of 14 $mJ \cdot cm^{-2}$ or 7 $mJ \cdot cm^{-2}$. For fluids with high UV absorption the flow rate of the liquid through the system is automatically slowed down, while for products with a lower absorption the flow rate is increased, so that the selected UV dose is consistently delivered to the product. In compliance with FDA regulations (FDA 2013), a turbulent flow regime (Re > 2200) was used at all tested conditions, and as an indicator of productivity, the flow rate of the juice through the UV unit was measured as the time required for processing a known volume of liquid.

Effect of SIS concentration

Two independent batches of freshly produced, non-pasteurized cloudy apple juice, which did not contain any preservatives or other additives, were purchased locally (Geneva, USA) and stored at 2 °C until used. Apple solids, as a slurry, were isolated during the screening step from the same processing line that produced the apple juice, and stored at 2 °C. Prior to use, apple solids were homogenized with a high shear mixer (model HSM-100 LSK, Charles Ross & Son Company, Hauppauge, USA). Solids were treated for 2 min with a disintegrating screen stator head, and 2 min with a slotted screen rotor head, at the maximum agitation speed of 10000 rpm.

Homogenized apple solids were added to the first batch of juice at different concentrations resulting in five solutions with turbidities from 1300 to 1700 NTU (refer as low turbidity apple juice) and to the second batch of apple juice obtaining six different solutions with turbidities from 1500 to 2800 NTU (refer as high turbidity apple juice).

A clear model solution was used to isolate the effect of the SIS concentration on the flow rate from the effect of the sample UV absorption due to the presence of colored compounds. Model solution was formulated considering the average chemical composition and physicochemical properties of cloudy apple juice reported by Markowski et al. (2009) and Piyasena et al. (2002). The composition of the solution is given in Table 6.

Table 6. Composition of the model apple juice solution used to assess the effects of concentration of SIS and SIS particle size on UV efficiency.

Component	Content (%)	Supplier
Distilled water	87.605	Available at the processing site
D-Fructose	6.147	Fisher Scientific (Fair Lawn, NJ)
Sucrose	3.242	Fisher Scientific (Fair Lawn, NJ)
D-Glucose	2.003	Fisher Scientific (Fair Lawn, NJ)
Malic acid	0.648	Presque Isle Wine Cellars (North East, PA)
Anhydrous sodium acetate	0.285	Mallinckrodt, Inc. (Paris, KY)
High methoxyl pectin	0.059	TIC Gums (Belcamp, NY)
Citric acid monohydrate	0.009	J.T. Baker Chemical Co. (Phillipsburg, NY)
Ascorbic acid	0.002	Growers Co-op Grape Juice Co. (Westfield, NY)

Apple solids added to the model solution were extracted at 1800 rpm using a turbo extractor for cold processing of apples, series CX 5 (Bertocchi SRL, Parma, Italy), equipped with a 1.5 mm screen, and immediately pasteurized at 96.6 °C for 6 minutes to prevent enzymatic browning. Solids were added to model solution resulting in six solutions with turbidities ranging from 3 to 1600 NTU. Apple juice and model solutions were treated at room temperature at a fixed UV dose of 14 $mJ \cdot cm^{-2}$ and the flow rate was determined. Processing trials were performed in triplicate.

To evaluate the effect of SIS on the inactivation of *E. coli* ATCC 25922, a non-pathogenic surrogate that has shown similar UV sensitivity to *E. coli* O157:H7 (Quintero-Ramos et al. 2004), the apple juice and model solutions used to evaluate the effect of SIS on flow rate were bottled and stored at 2 °C for up to 24 hours. Samples were inoculated and analyzed as indicated in the microbiological analysis section. Inoculated samples were UV treated using the "fixed flow rate" setting to avoid the automatic flow rate adjustment, thus UV exposure was variable depending on the sample's UV absorptivity. A fixed flow rate of approximately 214.5 $ml \cdot s^{-1}$ was used as it corresponds to the maximum pumping capacity of the *CiderSure* UV unit and therefore the minimum time of UV exposure achievable on this machine.

Effect of SIS particle size

Since no significant effect of SIS on the flow rate was observed at 14 $mJ \cdot cm^{-2}$ UV dose, the same effect was evaluated at a reduced dose of 7 $mJ \cdot cm^{-2}$ that still guarantees a turbulent flow regime. A clear model solution (Table 6) and apple solids with two

different average particle sizes were used to assess the effects of SIS concentration and particle size on the flow rate of the solution through the UV unit. Apple solids (average diameter of 895 μm) were obtained using a turbo extractor, following the procedure indicated above in the “Effect of SIS concentration” section. A second batch of solids, with a smaller average diameter (199 μm), was obtained by the same method but using a 0.4 mm screen. Solids were treated with steam at 99 ºC for 5 minutes, and a second homogenization step was carried out for 2 minutes with a high shear mixer model HSM-100 LSK (Charles Ross & Son Company, Hauppauge, USA) equipped with a high shear rotor/stator attachment. Solids were treated for additional 2 minutes with a fine screen stator head at 10000 rpm.

Apple solids, as slurries (with two different average particle sizes), were added to two independent model solutions resulting in turbidities from 4 to 500 NTU. The flow rate of the solution through the reactor was determined. After treated, samples were bottled and stored at 2 °C for up to 24 hours. Model solutions were then inoculated with *E. coli* ATCC 25922 and UV treated at a fixed flow rate of 214.5 $ml \cdot s^{-1}$. All treatments were executed in triplicate. Inoculation and analysis were performed as indicated in the microbiological analysis section.

Effect of time after apple pressing

Cloudy apple juice was prepared from three apple varietals: Jonagold, Golden Delicious and Rhode Island Greening. Apples were washed and passed through a comminuting machine (The W.J. Fitzpatrick Company, Chicago, USA), and immediately pressed in a

hydraulic rack-and-frame press for juice extraction. The resulting juice was treated at 14 mJ·cm^{-2} fixed UV dose after 0 to 120 min of apple pressing (10 to 20 min intervals). The flow rate and color of the juice were measured at each sampling time. The juice color was determined using the L', a' and b' Hunter color parameters, and the total color difference (*ΔE*) was calculated at each sampling time using the following equation:

$$\Delta E = \sqrt{\Delta L'^2 - \Delta a'^2 + \Delta b'^2}$$

After 120 min of apple pressing, juices were inoculated with *E. coli* ATCC 25922 and treated at 14 mJ·cm^{-2}. Treatments were performed in triplicate.

Physicochemical analyses

pH was measured using an Accumet Basic AB15 pH meter (Fisher Scientific, Pittsburgh, USA). Soluble solids content (degree Brix), were measured using a Leica Auto Abbe refractometer model 10500-802 (Leica Inc., Buffalo, USA). Titratable acidity (expressed as percentage of malic acid) was determined with a G20 compact titrator (Mettler Toledo, Schwerzenbach, Switzerland). Particle size distribution of apple solids was determined using a laser diffraction particle size analyzer Mastersizer 2000 (Malvern instruments, Worcestershire, UK). SIS concentration was estimated as percentage spin solids (Vaillant et al. 2008) by centrifuging 10 ml of pre-weighed homogenized sample at 2200 *g* for 15 min and draining the liquid.

Turbidity was measured using a HACH 2100P turbidimeter (Hach Company, Loveland, USA). A Hunter UltraScan XE spectrocolorimeter (Hunter Lab Assoc., Reston, USA) was used to measure color values L', a', and b'. The samples absorption coefficients (α)

were determined following the protocol reported by Koutchma et al. (2004), where α corresponds to the slope of the absorbance against the path length. After a 10-fold dilution in distilled water, samples absorbance was measured at 254 mm using a UV 1800 spectrophotometer (Shimadzu Scientific Instruments, Columbia, USA) equipped with demountable fused quartz cuvettes (NSG Precision Cells, Inc., Farmingdale, USA) of 0.1, 0.2, 0.5 and 1.0 mm path lengths. Moisture content of apple solids was determined with a moisture analyzer model MX-50 (A&D Co. Ltd., Tokyo, Japan).

Microbiological analysis

Quantification of *E. coli* was performed as reported by Basaran et al. (2004). *E. coli* ATCC 25922 (clinical isolate from the American Type Culture Collection) was obtained from the Food Microbiology Laboratory at the New York State Agricultural Experimental Station (Geneva, USA). A single isolated colony was transferred into 10 ml of Trypticase soy broth (TSB) (Difco, Becton Dickinson, Sparks, USA) and incubated for 5 ± 1 h at 35 ± 2 °C. The inoculated TSB was transferred into 400 ml of TSB and incubated for 20 ± 2 h at 35 ± 2 °C (to stationary phase) on a rotary platform shaker, at 250 rpm. Prior to UV treatment, approximately 1.8 l of each juice or model solution was inoculated with a 20 ml aliquot of *E. coli* suspension, resulting in an initial population of 10^6 to 10^7 CFU·ml^{-1}. Inoculated solutions were aseptically sampled before and after UV processing and poured-plated by duplicate using Trypticase soy agar (TSA). After incubation (20 ± 2 h at 35 ± 2 °C), colonies were counted and replicate counts were averaged. Microbial reduction was calculated as log (N/N_o) where N refers to the after treatment count and N_o to the initial count (CFU·ml^{-1}).

Statistical analyses

Linear regression analysis was used to evaluate the relationships between turbidity and spin solids and among the SIS concentration and flow rate of apple juice and model solutions. Analysis of variance (ANOVA) was used to test differences between mean flow rates and inactivation of *E. coli* within the five low turbidity apple juices, among the six high turbidity juices and within the model solutions. The same statistical analysis was used to compare the effect of turbidity level (low or high) on the juice flow rate and inactivation of *E. coli*. Means were further compared using Tukey's test. A multiple linear regression analysis was applied to assess the effect of SIS concentration and particle size on the flow rate of model solutions treated at 7 $mJ \cdot cm^{-2}$ UV dose. An interaction term between average particle size and turbidity was included to identify the effect of turbidity on the flow rate at different particle sizes. Statistical analyses were performed using JMP® version 11 (SAS Institute, Cary, USA). Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Relationship between turbidity and spin solids in cloudy apple juice

The SIS concentration was determined using two methods: turbidity and spin solids estimation. For apple juices with turbidities ranging from 1300 to 3200 NTU, a linear relationship ($r^2 = 0.93$) between the two measurements was found (Figure 7). Vaillant et al. (2008) reported that the relationship among these variables for different fruit juices (banana, pineapple, and blackberry) is slightly non-linear and can be expressed by a power law type equation. However, in that study the authors evaluated a wider range of

turbidities and reported equations with exponents lower than 1.5. Thus, considering the selected range of turbidity in our study, which comprises the typical turbidity values for commercial cloudy apple juice (Koutchma et al. 2004), the results obtained might represent a linear section of the curvilinear trend mentioned above. Bearing in mind that turbidity assessments allow getting immediate results, and that small variations in SIS content result in much higher variation in turbidity values, which makes the turbidity measurement more accurate in comparison with the spin solids estimation (Vaillant et al. 2008), turbidity was chosen as the preferred method to express the SIS concentration in the apple juice and model solutions for this study.

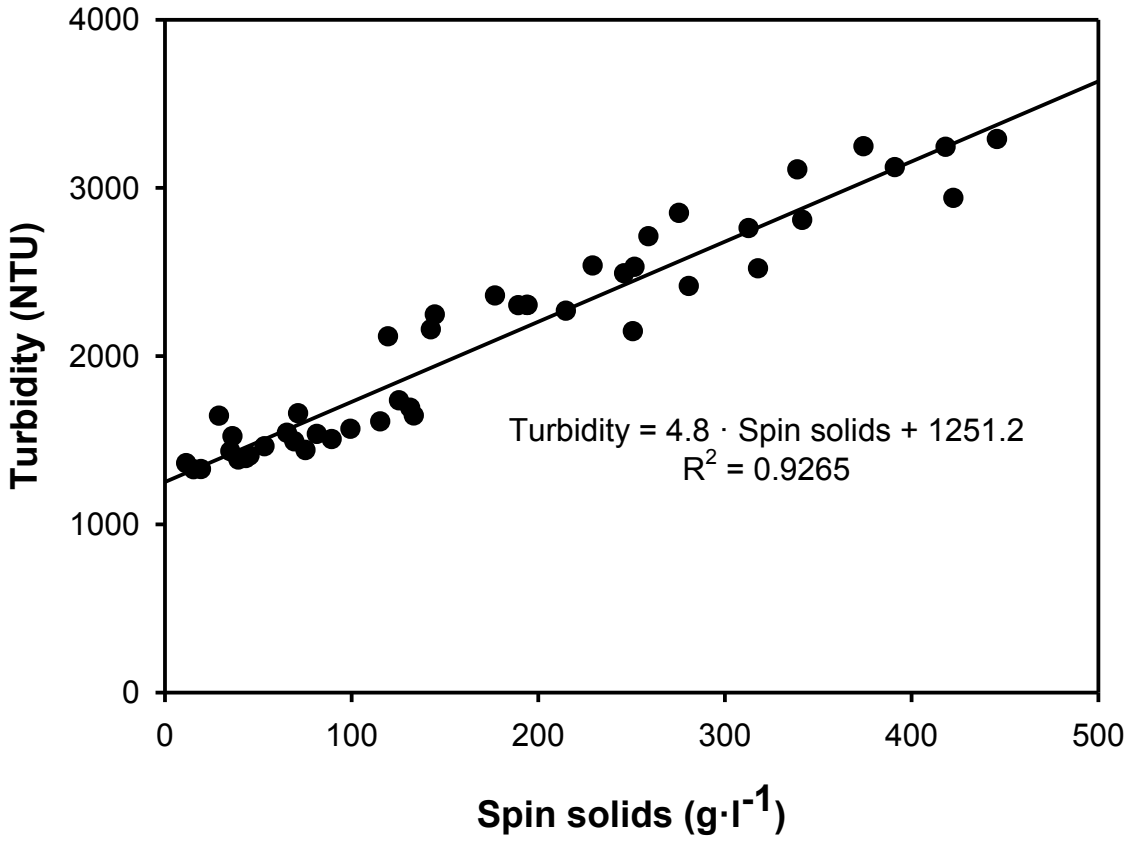

Figure 7. Turbidity as a function of spin solids concentration in cloudy apple juice.

Effect of SIS concentration

Physicochemical characterization of apple juice, model solution and apple solids are shown in Tables 7 and 8. When apple juice (six different juice samples with turbidities ranging between 3.8 ± 0.3 to 1612 ± 121 NTU) were treated at a fixed UV dose of 14 $mJ \cdot cm^{-2}$, the SIS concentration, in terms of turbidity, did not show a significant effect ($p > 0.05$) on the juice flow rate through the *CiderSure* (178 ± 13 $ml \cdot s^{-1}$). Moreover, when the treatment was applied under a "fixed flow rate", no significant differences ($p > 0.05$) on the log reduction of *E. coli* were observed (mean log reduction of 6.7 ± 0.3). Likewise, turbidity did not significantly affect ($p > 0.05$) the juice flow rate and inactivation of *E. coli* when the five low turbidity apple juices (from 1388 ± 12 to 1684 ± 45 NTU) were compared against each other, and within the six tested high turbidity apple juices (from 1526 ± 106 to 2800 ± 45 NTU). Average flow rates and log reductions in low and high turbidity apple juices are shown in Table 4. Nonetheless, when high and low turbidity apple juices were compared against each other, even though nonsignificant changes on the average flow rate were observed ($p > 0.05$), a significant difference in the mean log reductions of *E. coli* was found ($p < 0.05$) (Table 9). These differences are explained by the fact that, as reported by Parker & Darby (1995), higher concentrations of particulate matter cause a shielding effect for microorganisms limiting the effectiveness of the UV light technology. Moreover, solids could also absorb part of the UV light energy applied to the product and therefore reduce the amount of energy available for the inactivation of microorganisms (Murakami et al 2006).

Table 7. Physicochemical characterization of the liquid substrates used to evaluate the effect of SIS on the product flow rate and microbial inactivation of *E. coli* using UV treatment (mean ± standard deviations, n = 3).

Sample	pH	Soluble solids (ºBrix)	Titratable acidity (g malic acid·100 g^{-1})	Color			Turbidity (NTU)	Absorption coefficient (mm^{-1})
				L'	a'	b'		
Apple juice (low turbidity)	3.79 ± 0.01	12.29 ± 0.01	0.360 ± 0.001	30.01 ± 0.01	0.6 ± 0.1	2.7 ± 0.1	1381 ± 12	2.14 ± 0.07
Apple juice (high turbidity)	3.84 ± 0.04	13.6 ± 0.2	0.320 ± 0.001	28.0 ± 0.2	0.8 ± 0.2	3.37 ± 0.08	1526 ± 106	2.19 ± 0.04
Model solution	3.56 ± 0.01	11.28 ± 0.01	0.649 ± 0.002	43.16 ± 0.06	-0.65 ± 0.01	0.28 ± 0.02	3.80 ± 0.03	0.08 ± 0.02

Table 8. Physicochemical characterization of the apple solids used to evaluate the effect of SIS on the product flow rates and microbial inactivation of *E. coli* during UV treatment (mean ± standard deviations, n = 3).

Sample	pH	Soluble solids (ºBrix)	Moisture content (%)	Average particle diameter (μm)
Apple solids (low turbidity apple juice)	3.85 ± 0.03	11.5 ± 0.3	85 ± 1	417 ± 7
Apple solids (high turbidity apple juice)	3.78 ± 0.03	13.80 ± 0.02	85.1 ± 0.3	325 ± 2
Apple solids (model solution)	3.81 ± 0.01	12.43 ± 0.01	85.8 ± 0.2	905 ± 9

Table 9. Average flow rates and log reductions of *E. coli* ATCC 25922 for low and high turbidity apple juices treated with UV (mean ± standard deviations, n = 3).[a]

Sample	Average flow rate ($ml \cdot s^{-1}$)	Average log reduction
Apple juice (low turbidity)	64 ± 6[a]	5.55 ± 0.04[b]
Apple juice (high turbidity)	69 ± 7[a]	4.8 ± 0.7[a]

[a] Means with the same letter within the same column are not significantly different (Tukey's test $p > 0.05$).

Expected differences between the mean flow rates of apple juice and model solutions were observed and can be explained by the disparities in the apparent absorption coefficients of the samples (Table 9), confirming that UV light transmission through liquid food products decreases with increasing UV absorbance (Koutchma et al. 2004; Sharma 1992; Sommer et al. 1995).

Effect of SIS particle size

In model solutions treated at 7 $mJ \cdot cm^{-2}$ UV dose, a negative linear relationship between turbidity and flow rate was found (Figure 8). This effect was observed in the two independent batches of model solutions, where apple solids with two different average particle sizes were added. A significant interaction between average particle size and turbidity was also observed ($p < 0.05$). Therefore, particle size has a significant effect on the flow rate when turbidity changes. Furthermore, increments in turbidity due to the addition of larger particles caused a more pronounced decrease on the flow rate as compared to model solutions where smaller particles were added (Figure 8). For clear solutions containing the larger solids (average particle diameter of 895 μm), increments

in the concentration of SIS explained about 98% of the total linear reduction in the flow rate whereas for solutions containing smaller solids (average particle diameter of 199 µm) the coefficient of determination was 87%.

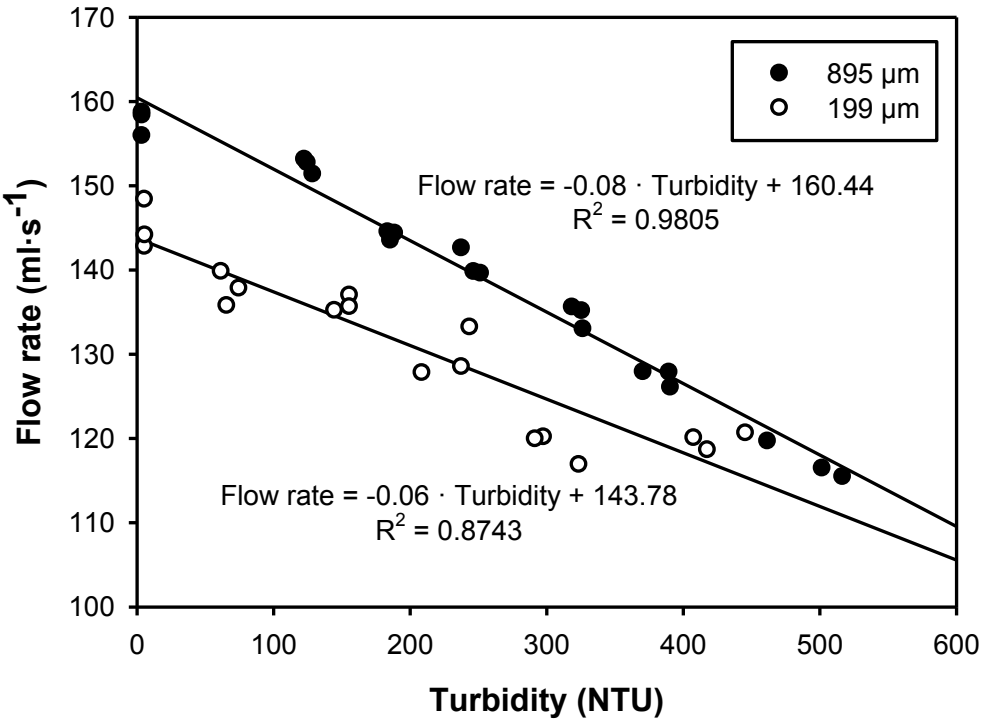

Figure 8. Flow rate as a function of turbidity of model solutions with added apple solids with two different average particle diameters (895 µm and 199 µm), and treated at 7 mJ·cm^{-2} fixed UV dose.

Considering that the most adverse effect of solids on flow rate was observe in presence of larger particles, the log inactivation of *E. coli* was tested in those samples and, regardless of the solids concentration, a greater than 5-log reduction of *E. coli* was achieved when the treatment was applied using the "fixed flow rate" mode. No significant differences ($p > 0.05$) in the log reduction were found (average log reduction of 6.4 ± 0.5). These results suggest that despite the presence of larger particles, the turbulent flow regime used at all tested conditions (Reynolds numbers between 3000 to

4000) ensured a uniform distribution of the UV light during the application of the treatment.

Effect of time after apple pressing

The characterization of apple juices used to evaluate the effect of time after apple pressing is shown in Table 10. An attempt was made to record the absorption coefficient (α) of juices at each sampling time but the method was slow enough that samples were already browned when measured. Therefore, the total change in color (*ΔE)* based on the Hunter color parameters was calculated (Table 11). For Jonagold juice, an important reduction in the flow rate of about 17% (from 197 ± 6 $ml \cdot s^{-1}$ to 162 ± 7 $ml \cdot s^{-1}$) was found within the first 60 minutes after apple pressing. Afterwards, no apparent differences were observed on the flow rate, which on average was 160 ± 6 $ml \cdot s^{-1}$ (Figure 9). A similar, but not as strong, effect was observed for the Rhode Island Greening and Golden Delicious varietals (Figure 9).

A multiple linear regression analysis showed that a linear relationship between time after pressing, after a natural log transformation, and flow rate was found for every varietal. A significant interaction between varietal and time was also observed ($p < 0.05$). The decreasing trend of the flow rate was more pronounced for Jonagold, followed by Golden Delicious and less marked for the Rhode Island Greening varietal. Moreover, the initial flow rates of each varietal differed significantly ($p < 0.05$) in the same order: highest for Jonagold and lowest for Rhode Island Greening. These differences can be explained by the observed disparities in the initial turbidity of the

samples (Table 10). Thus, at lower turbidity, a higher flow rate was obtained, as expected.

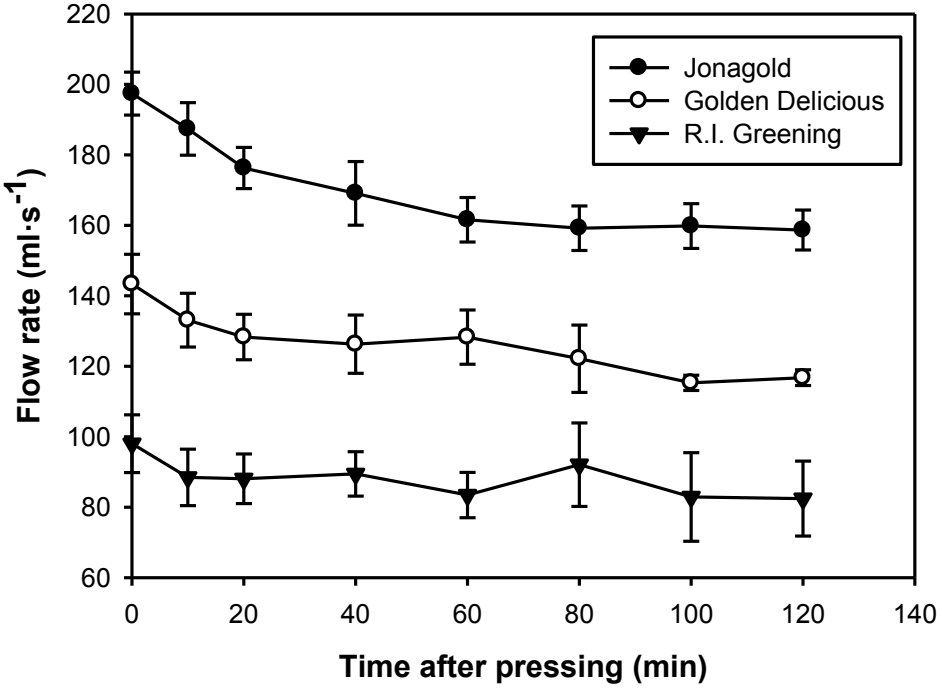

Figure 9. Flow rate as a function of time after pressing for apple juice made from three apple varieties (error bars represent standard deviations, n = 3).

The total color change significantly increased after apple pressing for Rhode Island Greening juice ($p < 0.05$) (Table 11). Same trend was observed for juices made from Jonagold and Golden Delicious but, for the last two varietals, the change was nonsignificant ($p > 0.05$). Thus, darkening of apple juice, mostly due to enzymatic reactions, can reduce the efficiency of UV treatments in terms of flow rate, and this effect is apple varietal dependent. Yet, regardless of the varietal tested, at 14 $mJ \cdot cm^{-2}$ UV dose, a greater than 5-log reduction of *E. coli* was achieved when inoculated samples where UV pasteurized at 120 min after apple pressing, where no further changes in color were observed.

Table 10. Physicochemical characterization of the apple juices used to evaluate the effect of time after apple pressing on the flow rate and microbial inactivation of *E. coli* by UV treatment (mean ± standard deviations, n = 3).

Apple variety	pH	Soluble solids (°) Brix	Titratable acidity (g malic acid/100 g)	Turbidity (NTU)	Color		
					L’	a'	b'
Jonagold	3.59 ± 0.07	11.7 ± 0.4	0.45 ± 0.02	628 ± 61	28.7 ± 0.2	0.0 ± 0.1	0.04 ± 0.08
Golden Delicious	3.39 ± 0.02	11.6 ± 0.4	0.24 ± 0.08	872 ± 53	29.3 ± 0.1	0.7 ± 0.6	1.4 ± 0.6
Rhode Island Greening	3.43 ± 0.05	12.6 ± 0.1	0.70 ± 0.04	1400 ± 90	29.4 ± 0.5	0.3 ± 0.1	1.1 ± 0.2

Table 11. Total change in color for the varietal apple juices used to evaluate the effect of time after apple pressing on the juice flow rate through the UV unit (mean ± standard deviations, n = 3).

Time after pressing (min)	Total change in color *(ΔE)*		
	Jonagold	Golden Delicious	Rhode Island Greening
10	2 ± 1	0.5 ± 0.7	1.05 ± 0.08
20	2 ± 1	0.6 ± 0.6	1.2 ± 0.08
40	2.0 ± 0.7	0.8 ± 0.6	1.4 ± 0.1
80	2.3 ± 0.3	0.9 ± 0.5	1.6 ± 0.2
80	2.3 ± 0.2	0.9 ± 0.5	1.8 ± 0.1
100	2.2 ± 0.3	0.9 ± 0.5	1.9 ± 0.08
120	2.2 ± 0.3	1.0 ± 0.5	1.9 ± 0.08

CONCLUSIONS

Although a greater than 5-log reduction of *E. coli* was achieved under all tested conditions, considering the observed negative effects of increasing the SIS concentration and particle size in UV-treated cloudy apple juice, special considerations must be given to these variables when this nonthermal technology is used for treating this and similar liquid food products. Furthermore, since increasing the time after apple pressing was associated with lower juice flow rates; coupling the juice extraction system with the UV reactor would be recommended to minimize this undesirable effect. However factors such as the juice extraction system, production volume, and blend of apple varieties should be considered before the implementation of this strategy.

The results obtained in this study will assist current producers of UV-treated apple juice and similar liquid food products to understand how turbidity, particle size of SIS, and time after apple pressing may influence the productivity of UV systems when using a commercial-scale processing reactor such as the *CiderSure*. Our findings may also assist process developers and researchers to optimize the application of this nonthermal technology and to design new UV-based processes for products containing an important amount of particulate matter and colored compounds.

ACKNOWLEDGEMENTS

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CHAPTER 5

EFFECT OF ASCORBIC ACID AND SELECTED PRESERVATIVES ON THE EFFICIENCY OF ULTRAVIOLET TREATMENT OF APPLE JUICE AT A FIXED UV DOSE OF 14 mJ·cm^{-2}

ABSTRACT

The efficiency of UV pasteurization of apple juice containing selected additives was evaluated. Ascorbic acid, total vitamin C, sodium benzoate, potassium sorbate, and sulfur dioxide concentrations as well as juices' absorption coefficients were measured before and after UV at a fixed dose of 14 mJ·cm^{-2}. The juice flow rate through the UV reactor was determined. UV treated samples containing ascorbic acid and inoculated with *Escherichia coli* ATCC 25922 (10^7 CFU·ml^{-1}) were analyzed for microbial reduction. Adding ascorbic acid, sorbate, and benzoate significantly increased juice absorption coefficients and decreased flow rate ($p < 0.05$). UV showed no effect on total vitamin C and benzoate concentrations ($p > 0.05$) but negatively affected sulfur dioxide, ascorbic acid, and particularly sorbate levels ($p < 0.05$). Increases in ascorbic acid concentrations decreased *E. coli* inactivation ($p < 0.0001$). Thus, additives that adversely influence UV efficiency or are degraded should be added after treatment.

INTRODUCTION

Since the recognition of ultraviolet (UV) light treatment as an alternative for the thermal pasteurization of beverages (FDA, 2013a), this technology has become a viable nonthermal processing option for these products. The high efficiency of pathogen

reduction (Basaran et al., 2004; Hanes et al., 2002; Oteiza et al., 2005; Oteiza et al., 2010; Quintero-Ramos et al., 2004), and the reduced loss of nutritional components accompanied by fewer unwanted physical and chemical changes (Caminiti et al., 2010; Tran and Farid, 2004) are some of the advantages that have attracted the attention of consumers, producers, and researchers towards this technology. However, previous studies have suggested that UV applications might be limited for treating selected beverages due to the presence of compounds that strongly absorb UV light (Koutchma et al., 2004; Koutchma et al., 2007; Oteiza et al., 2005).

Research has shown that vitamin C, a naturally occurring and commonly added nutrient in juices, may dramatically decrease UV light effectiveness in different fruit juices by diminishing the inactivation rates of certain microorganisms such as *E. coli* (Koutchma et al., 2004; Koutchma, 2008; Oteiza et al., 2005). Furthermore, this light-sensitive nutrient might be severely degraded during UV pasteurization. Koutchma et al. (2002) reported a destruction of vitamin C ranging from 30 to 40% when apple juice was exposed to a 600 $mJ \cdot cm^{-2}$ UV dose, and when exposed to a similar UV dosage, a degradation of 18% and 25% in orange and carrot juices, respectively. Similarly, Tran and Farid (2004) revealed a vitamin C concentration decline of 17% in orange juice treated with a 100 $mJ \cdot cm^{-2}$ UV dose. Contradictorily, no significant difference in ascorbic acid concentration was found when apple cider was treated for seven consecutive passes (accumulative UV dose of 98 $mJ \cdot cm^{-2}$) using a commercial UV apparatus, under a turbulent flow regime, and at a 14 $mJ \cdot cm^{-2}$ UV dose per pass (Assatarakul et al., 2011; Dong et al., 2010).

The addition of other additives such as preservatives is also thought to increase the absorptivity of beverages and consequently limit the performance of UV. Nevertheless, no published information regarding this effect in juices is currently available. Considering that UV treated beverages are not shelf-stable products, and that the addition of preservatives represents a viable hurdle approach to preserve their quality and ensure an extended shelf life, it becomes relevant to evaluate if these compounds may adversely affect the efficiency of this treatment. Furthermore, in the particular case of potassium sorbate, a preservative commonly used in juices and beverages, Cigić et al. (2001) previously found that, in water, this additive isomerizes under UV radiation after a 20 minute exposure to a 50 W high-pressure mercury lamp, and that the resultant mixture of isomers had lower antimicrobial activity than the original *trans-trans* isomer. However, this phenomenon has not been studied in UV treated juices or using commercial UV juice processing units.

Considering the relevance of understanding the effect of certain food additives on the productivity and efficiency of UV processing systems, and the potential influence of UV radiation on the stability of those compounds, the present study sought to evaluate both effects in apple juice containing ascorbic acid, as well as sodium benzoate, potassium sorbate, and sulfur dioxide, the most common antimicrobials used for fresh juice preservation (Basaran-Akgul et al., 2009).

2. MATERIALS AND METHODS

2.1 Reagents

1,4 dithiothreitol (DTT) was purchased from J.T. Baker (Center Valley, PA). L (+) ascorbic acid, stabilized metaphosphoric acid (MPA), high performance liquid chromatography (HPLC) grade acetonitrile, monobasic potassium phosphate, phosphoric acid, sodium benzoate, potassium sorbate, and sulfuric acid were obtained from Fisher Scientific (Fair Lawn, NJ). Trypticase soy broth (TSB) was purchased from Difco, Becton Dickinson (Sparks, MD).

2.2 Apple juice

Locally purchased apple juice concentrate was reconstituted with distilled water to 12 °Brix, pasteurized at 73.9 °C for 6 seconds in an UHT/HTST Lab-25 HV tubular heat exchanger (MicroThermics Inc., Raleigh, NC), and kept refrigerated at 4 °C for up to two days and until used.

2.3 UV radiation processing unit

UV treatments were carried out in a commercial *CiderSure* 3500 UV juice processing unit (FPE Inc., Macedon, NY) at a wavelength of 254 nm. This UV machine was previously validated to ensure a greater than 5-log reduction of *E. coli* O157:H7 and *Cryptosporidium parvum* in apple cider (Basaran et al., 2004; Hanes et al., 2002). The *CiderSure* reactor comprises two UV light sensors that measure juice transmittance. Based on the measurements, the machine automatically adjusts the flow rate, guaranteeing a fixed UV dose of 14 $mJ \cdot cm^{-2}$. A thorough technical description of the

design and operation of this UV juice-processing units has been published by Basaran et al. (2004).

2.4 Sample preparation and UV processing

Apple juice containing various concentrations of either ascorbic acid (0-600 $mg \cdot kg^{-1}$), potassium sorbate (0-200 $mg \cdot kg^{-1}$), sodium benzoate (0-1000 $mg \cdot kg^{-1}$), or sulfur dioxide (0-280 $mg \cdot kg^{-}1$, corresponding to a concentration of free sulfur dioxide ranging from 0-160 $mg \cdot kg^{-1}$) were UV treated at 14 $mJ \cdot cm^{-2}$ fixed UV dose in a single-pass treatment. All additives were added in concentrations that comply with the levels indicated by the U.S. Food and Drug Administration (FDA, 2013b).

Flow rates were determined for all treatments using the bucket and stopwatch method, where the time required for filling a known volume of UV treated juice was measured. Samples before and after UV treatment were collected in amber high-density polyethylene (HDPE) centrifuge tubes and, excluding ascorbic acid trials, and stored at 4 °C until the analyses were performed. Samples from trials involving ascorbic acid and total vitamin C were analyzed via HPLC immediately after the addition of ascorbic acid to the juice and the application of the UV treatment.

Total vitamin C, ascorbic acid, potassium sorbate, sodium benzoate, and free and total sulfur dioxide concentrations as well as the apparent absorption coefficient of all juice samples were determined before and after UV treatments.

In order to evaluate if the bleaching effect caused by the addition of sulfur dioxide, a phenomenon previously reported in the literature (Joslyn and Braverman, 1954), has a significant effect on the flow rate, the Hunter color parameters of the solutions containing this preservative were measured before UV treatment.

To examine the potential degradative effect of UV exposure on potassium sorbate, apple juice containing 100 $mg \cdot kg^{-1}$ of the additive was subjected to 5 consecutive passes (a cumulative UV dose between 0 and 70 $mJ \cdot cm^{-2}$), and the residual sorbate concentration was measured after each pass.

To assess the effect of ascorbic acid concentration on the log reduction of *E. coli* ATCC 25922, two independent batches of reconstituted apple juice containing ascorbic acid between 0 and 600 $mg \cdot kg^{-1}$ were inoculated at 10^7 $CFU \cdot ml^{-1}$, and subjected to two independent treatments: (1) fixed flow rate of 214.5 $ml \cdot s^{-1}$ which corresponds to the maximum pumping capacity of the *CiderSure* and therefore the minimum time of UV exposure in this UV unit, and (2) fixed UV dose of 14 $mJ \cdot cm^{-2}$ with automatic flow rate adjustment. The *E. coli* counts, before and after treatment, were determined following the protocol detailed in section 2.7. All trials were conducted in triplicate.

2.5 Total vitamin C and ascorbic acid determination

Ascorbic acid and total vitamin C concentration, defined as the sum of ascorbic acid and its oxidized form dehydroascorbic acid (DHA), were determined via HPLC with a modified version of the protocol described by Margolis et al. (1990). For total vitamin C

quantification, the DHA was reduced to ascorbic acid by the addition of dithiothreitol, and eventually measured in conjunction with the native and residual ascorbic acid present in the juice.

A 50 mmol potassium phosphate monobasic solution, adjusted to a pH of 2.8 by the addition of phosphoric acid, was used as the mobile phase. For the standard curve preparation, a stock solution of ascorbic acid at 5000 $mg \cdot kg^{-1}$ was used to prepare standard solutions at 25, 100, 200, 300, 400, 500 and 600 $mg \cdot kg^{-1}$ in HPLC-grade water. One ml of each standard solution was diluted by adding 400 μl of 5 $mg \cdot ml^{-1}$ DTT, 200 μl of 4% MPA, and 400 μl of HPLC-grade acetonitrile. Diluted standards were filtered into amber autosampler vials with a nylon syringe filter (13 mm × 0.45 μm pore size; Krackeler Scientific, Albany, NY), and analyzed by HPLC. The calibration standard curve was performed in triplicate at the beginning of the experiment and eventually used to determine the total vitamin C and ascorbic acid concentrations in samples.

Standard solutions and samples were injected onto a Thermo Scientific Aquasil C_{18} endcapped column (250 mm x 4 mm id, 5 μm particle size, 100 nm pore size; Thermo Scientific, Waltham, MA) and resolved at a 1 $ml \cdot min^{-1}$ flow rate in an isocratic run for 20 min at ambient column temperature (22-25 ºC) and with a detection wavelength of 254 nm. One ml of each sample was diluted in a 2.2 ml vial with 400 μl of 5 $mg \cdot ml^{-1}$ DTT; vials were capped and vortex-mixed for 15 s. After 1 hour of storage at room temperature in dark conditions, 200 μl of a 4% MPA solution and 400 μl of acetonitrile were added. Samples were vortex-mixed and centrifuged at 1000 × g for 30 minutes at

4 ºC. The supernatant fluid was filtered as the standards and analyzed on an Agilent 1100 series HPLC (Santa Clara, CA).

The ascorbic acid quantification was performed following the same procedure indicated for total vitamin C except that the addition of DTT and the incubation time after the addition of the reagent were omitted.

2.6 Potassium sorbate and sodium benzoate determination

A 20% HPLC-grade acetonitrile in a 0.01 N sulfuric acid solution was used as the mobile phase. For the calibration standard curve, a combined stock solution of potassium sorbate and sodium benzoate at a concentration of 1000 $mg \cdot kg^{-1}$ of each reagent were used for preparing standard solutions at 2, 5, 10, 20, 50 and 100 $mg \cdot kg^{-1}$ for each compound. Standards were filtered as the standards used for total vitamin C quantification. The calibration standard curve was performed in triplicate.

A Bio-Rad Aminex HPX-87H column fitted with a micro-guard cation H refill cartridge (Bio-Rad, Hercules, CA) was used at 0.6 $ml \cdot min^{-1}$ flow rate in an isocratic elution over 30 min at a column temperature of 60 °C. In the case of potassium sorbate determination, a detection wavelength of 260 nm was used whereas 230 nm was selected for sodium benzoate analyses. After a 10-fold dilution of the samples using HPLC-grade water, juices were filtered as the standards and analyzed in an Agilent 1100 series HPLC.

2.7 Free and total sulfur dioxide determination

A multi-channel segmented-flow analyzer (FIA) was used for the automatic determination of free and total sulfur dioxide. The system comprises a FIAstar^{TM} 5000 (FOSS, Höganäs, Sweden) wine analyzer and an autosampler that operate by the SoFIA software (service pack 3). This automatic system provides results for free and total sulfur dioxide concentrations that are not significantly different from traditional measurement methods (Barril et al., 2012). A sample of additive-free apple juice was used as the blank and all measurements were performed in triplicate.

2.8 Microbiological analysis

E. coli ATCC 25922, a non-pathogenic surrogate with similar UV sensitivity than pathogenic *E. coli* O157:H7 (Quintero-Ramos et al., 2004), was obtained from the Food Microbiology Laboratory at the New York State Agricultural Experiment Station (Geneva, NY). A single colony of the strain was transferred into 5 ml of TSB and grown for 5 ± 1 h at 35 ± 2 ºC. The cell culture was then transferred into 500 ml of TSB and incubated overnight for 20 ± 2 h at 35 ± 2 ºC on a rotatory platform shaker (New Brunswick Scientific Co., Edison, NJ) at 250 rpm. Samples of approximately 1.8 L of reconstituted and pasteurized apple juice were inoculated with 20 ml of the bacterial solution resulting in an initial population of 10^7 CFU·ml^{-1}. Apple juice was aseptically sampled and analyzed before and after UV processing. Samples were subjected to seven serial dilutions in 9 ml of sterile 0.1% peptone water and each dilution was plated in duplicate. Petri dishes were pour-plated with Trypticase soy agar and incubated for

20 ± 2 h at 35 ± 2 °C. The log reduction of *E. coli* was calculated as the difference between the log-transformed counts before and after the UV treatment.

2.9 Physicochemical characterization

The pH, total titratable acidity (expressed as grams of malic acid per 100 ml of apple juice), soluble solids content (reported as degrees Brix), turbidity, color, and the apparent absorption coefficient of the reconstituted juice samples were measured. The pH was determined using a standard calibrated Accumet Basic AB15 pH meter (Fisher Scientific, Pittsburgh, PA). Soluble solids contents were measured with a Leica Auto Abbe refractometer model 10500-802 (Leica Inc., Buffalo, NY). Total titratable acidity was estimated using a G20 compact titrator (Mettler Toledo, Schwerzenbach, Switzerland). Turbidity measurements were performed in a HACH 2100P portable turbidimeter (Hach Company, Loveland, CO). Hunter color parameters L', a', and b' were determined using the reflectance-specular included (RSIN) mode in a Hunter UltraScan XE spectrocolorimeter (Hunter Lab Assoc., Reston, VA). The juice apparent absorption coefficient (α) was calculated following the protocol described by Koutchma et al. (2004), where α corresponds to the slope obtained from plotting the sample absorbance against the path length. After a 10-fold dilution in distilled water, the sample absorbance was measured at 254 mm with a UV-1800 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) equipped with demountable fused quartz cuvettes of 0.1, 0.2, 0.5 and 1.0 mm path length (NSG Precission Cells, INC., Farmingdale, NY). All physicochemical determinations were executed in triplicate.

2.10 Statistical analyses

Multiple linear regression analyses were used to assess the existent relationship between the concentration of ascorbic acid, potassium sorbate, sodium benzoate, and sulfur dioxide, and the juice apparent absorption coefficient, as well as the flow rate of the juice through the UV machine during processing. This analysis was also used to determine the effect of the UV treatment on the juice's absorption coefficients and concentrations of the additives tested.

The effect of adding sulfur dioxide on apple juice's L', a', and b' color attributes was determined using analysis of variance (ANOVA). ANOVA was also used to determine the effect of the UV dose on the stability of potassium sorbate in terms of concentration of the additive, and the effect of ascorbic acid concentrations on the log reduction of *E. coli* ATCC 25922. Means were further compared using Tukey's honestly significant difference (HSD) test at a significance level of 0.05. All statistical analyses were performed using JMP® version 10 (SAS Institute, Cary, NC).

3. RESULTS AND DISCUSSION

3.1. Effect of additives on juice apparent absorption coefficient and flow rate during UV processing

Table 11 shows the physicochemical characterization of apple juices used to determine the effect of the selected additives on the juices' apparent absorption coefficients and the *CiderSure* flow rates, and to assess the impact of UV radiation on the stability of those compounds.

Table 11. Physicochemical characterization of reconstituted apple juices before the addition of additives (mean ± standard deviation, n = 3).

Additive	pH	Soluble solids (ºBrix)	Titratable acidity (%)	Color			Turbidity (NTU)	Apparent absorption coefficient (mm^{-1})
				L’	a'	b'		
Ascorbic acid	3.61 ± 0.03	12.8 ± 0.2	0.40 ± 0.02	31.1 ± 0.3	5.3 ± 0.3	6.7 ± 0.5	18 ± 3	1.5 ± 0.2
Potassium sorbate	3.67 ± 0.01	12.93 ± 0.02	0.40 ± 0.02	30.7 ± 0.1	5.5 ± 0.2	7.1 ± 0.1	18 ± 1	2.00 ± 0.06
Sodium benzoate	3.56 ± 0.01	12.89 ± 0.04	0.375 ± 0.002	30.713 ± 0.006	5.4 ± 0.1	7.1 ± 0.3	26.5 ± 0.7	1.94 ± 0.04
Sulfur dioxide	3.30 ± 0.01	12.37 ± 0.03	0.63 ± 0.04	55.1 ± 0.2	4.69 ± 0.05	36.8 ± 0.03	7.0 ± 0.1	0.81 ± 0.07

Linear relationships between ascorbic acid, potassium sorbate, and sodium benzoate concentrations, and the juices' apparent absorption coefficients were observed (Figure 10). Only in the case of potassium sorbate a significant effect of the UV treatment on the relationship between concentration and measured absorption coefficient was observed. This will be further discussed in section 3.2.

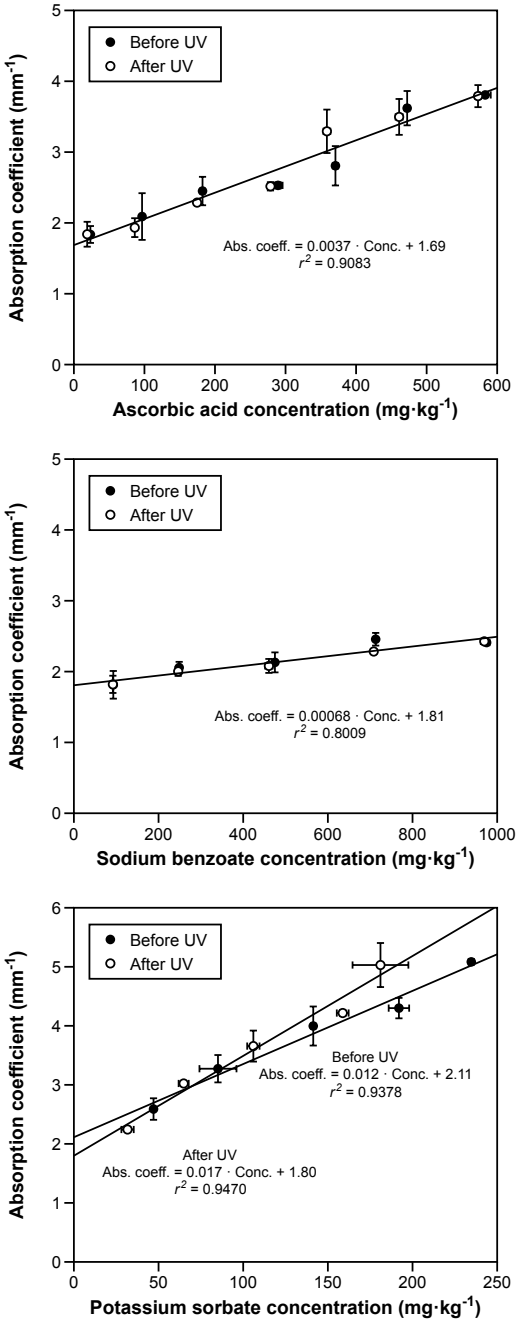

Figure 10. Apparent absorption coefficient at 254 nm, before and after UV treatment, as a function of the concentrations of the selected additives (n = 3, error bars show standard deviation).

Accordingly, an increase in the concentrations of these three additives resulted in a negative effect on the flow rates measured during UV processing (Figure 11). The reported changes in flow rate are explained by the fact that the *CiderSure* 3500 UV reactor is equipped with two UV light sensors that measure the UV energy transmitted through the treated liquid food product every 50 ms and, based on those measurements, the UV apparatus has been programmed to automatically adjust the pump flow rate ensuring a constant UV dose of 14 $mJ \cdot cm^{-2}$; this condition has been proven effective in achieving a greater than 5-log reduction of *E. coli* O157:H7 and *C. parvum* in apple cider (Basaran et al., 2004; Quintero-Ramos et al., 2004). Therefore, for solutions with high UV absorption, the machine reduces the product flow while for liquids with low absorption coefficients the pumped flow rate is increased. In both cases, a constant UV exposure is guaranteed.

A multiple linear regression analysis of the effect of the square root of the concentration of additives on the flow rate (Figure 11) showed that increases in concentrations of potassium sorbate and ascorbic acid caused a more pronounced decrease in the flow rate when compared to sodium benzoate. Moreover, no significant differences in flow rate reduction were confirmed in samples containing increasing concentrations of potassium sorbate and ascorbic acid (nonsignificant differences in their respective slopes, $p = 0.21$). The resulting model presented a coefficient of determination (r^2) of 96%.

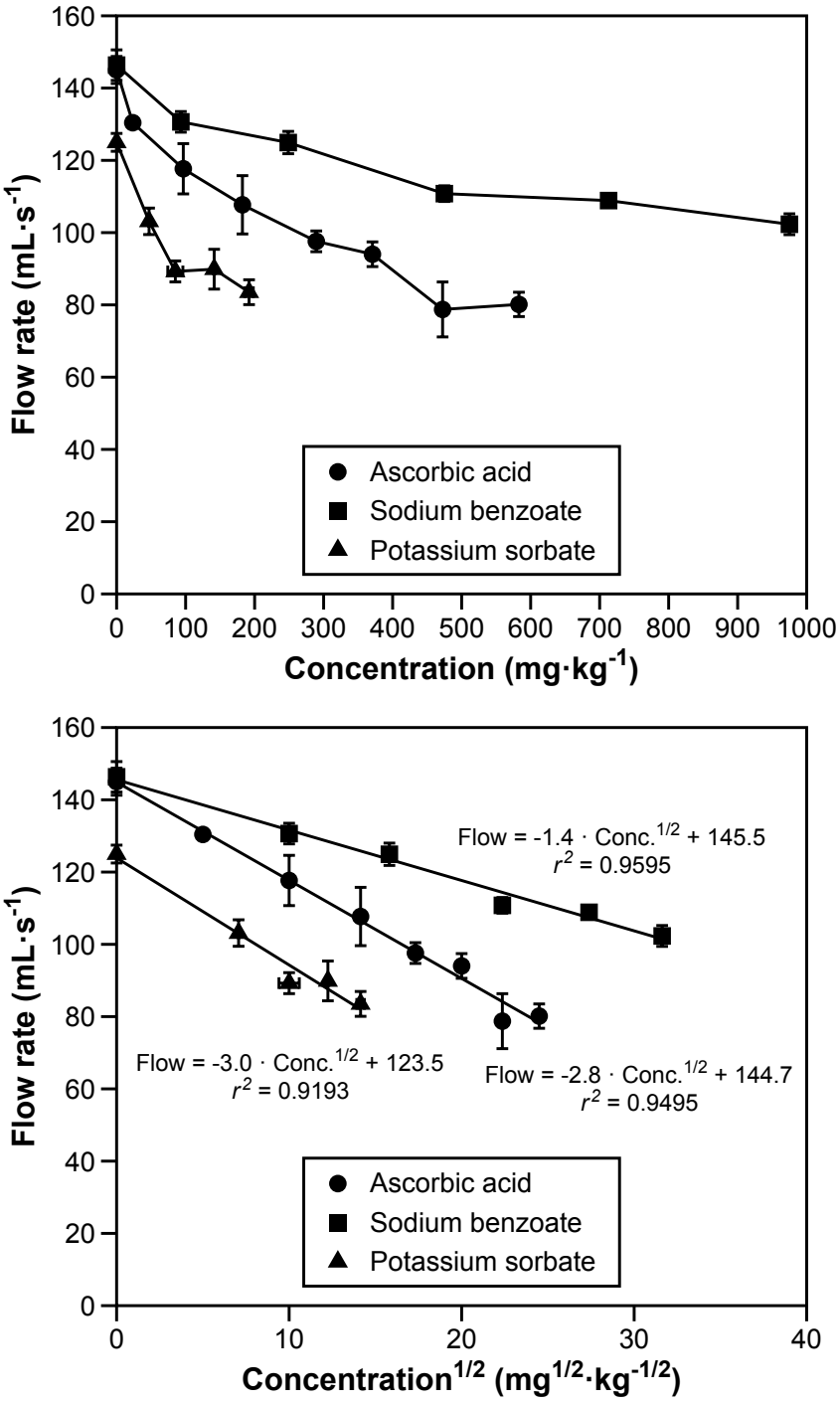

Figure 11. Flow rate as a function of the concentration and the square root of the concentration of selected additives in apple juice, treated with a 14 mJ·cm^{-2} UV dose (n = 3, error bars show standard deviation).

The juice's apparent absorption coefficient was not significantly altered by the addition of sulfur dioxide (p = 0.37) and therefore the flow rate (196 ± 4 ml·s^{-1}) was not adversely affected in samples containing increasing levels of this preservative (p = 0.66).

Table 12. Apple juice color parameters in samples containing increasing concentrations of free sulfur dioxide measured before UV radiation at 14 mJ·cm^{-2} UV dose (mean ± standard deviation, n = 3).[a]

Free sulfur dioxide – nominal concentration (mg·kg^{-1})	L'	a'	b'
0	55.1^{c} ± 0.2	4.69^{a} ± 0.05	36.8^{b} ± 1
60	56.5^{ab} ± 0.3	3.60^{b} ± 0.04	37.4^{ab} ± 0.7
75	56.4^{b} ± 0.1	3.50^{c} ± 0.02	37.2^{ab} ± 0.5
110	56.8^{ab} ± 0.3	3.38^{d} ± 0.03	37.5^{ab} ± 1
130	57.3^{ab} ± 0.4	3.37^{d} ± 0.02	37.6^{ab} ± 0.2
160	57.4^{a} ± 0.4	3.27^{e} ± 0.03	37.7^{a} ± 1

[a] Means not followed by the same superscript within the columns are significantly different (p < 0.05).

Although the addition of sulfur dioxide did not affect the juice's apparent absorption coefficient, the evaluated concentrations of free sulfur dioxide (0 to 160 mg·kg^{-1}) caused a significant rise in lightness (p = 0.0001) and the b' color parameter (p = 0.02). Also, a significant detrimental effect on the a' color parameter (p = 0.0001) was found (Table 12). In agreement with our findings, Basaran-Akgul et al. (2009) previously described a lightening phenomenon of apple cider due to the addition of sulfur dioxide. Accordingly, Roberts and McWeeny (1972) postulated that these changes are explained by a bleaching action of the preservative upon non-enzymatic browning pigments. Despite the fact that sulfur dioxide leads to significant changes in color, the observed variations in the visible spectrum did not impact the samples' absorption coefficients,

explaining the reported nonsignificant changes on the flow rate when juices containing this preservative were UV treated.

3.2. Effect of UV radiation on the stability of the selected additives

The apparent absorption coefficients of samples with increasing concentrations of ascorbic acid, sodium benzoate (Figure 10), and sulfur dioxide were not significantly affected by the single-pass UV process ($p > 0.05$). On the other hand, changes were observed in juices containing potassium sorbate, where a marked effect of the UV treatment on the juices' apparent absorption coefficients was observed, depicted as a significant increase ($p = 0.0003$) in the slope of the relationship between potassium sorbate concentration and absorption coefficient after UV pasteurization (Figure 10).

As for the concentrations of the selected additives, total vitamin C and sodium benzoate concentrations were not adversely affected by the UV treatment ($p > 0.05$). However, the concentrations of free sulfur dioxide and ascorbic acid slightly decreased after UV processing, as indicated by significant differences in the linear relationships between nominal and measured concentrations before and after the UV (Figure 12). These differences were characterized by significantly different intercepts for ascorbic acid ($p = 0.0005$); and intercepts ($p = 0.0001$) and slopes ($p = 0.007$) for the case of free sulfur dioxide.

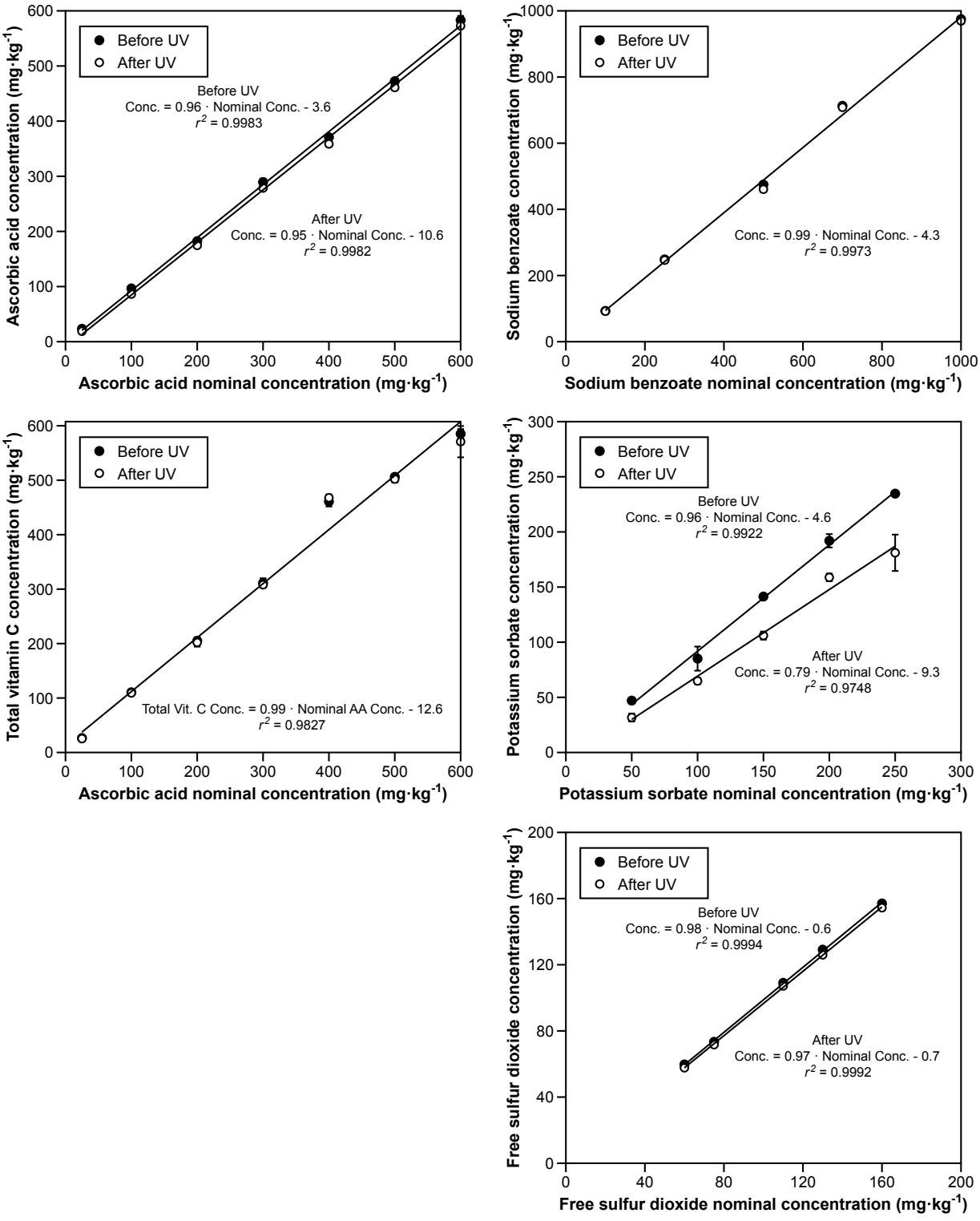

Figure 12. Effect of UV on the concentration of selected additives in apple juice.

Sulfur dioxide is used by the food industry as a preservative and antioxidant and it occurs in two different forms in juices: free (inorganic forms including SO_2 and HSO_3) and bound (fixed to organic compounds). Total SO_2 represents the sum of all species of free and bound SO_2 (Bartroli et al., 1991; Čmelík et al., 2005). Considering that the free sulfur dioxide corresponds to the active fraction in terms of preventing excessive oxidation and microbial development (Santos et al., 2012), the effect of the UV pasteurization over this preservative was reported in this study based on that form (Figure 12). However, the total sulfur dioxide content was also determined and the same trend reported for the free form was consistently observed (data not shown). Total sulfur dioxide concentration slightly decreased after UV pasteurization when nominal and measured concentrations were compared, showing significantly different slopes (p = 0.046).

Chemical and biochemical degradation of ascorbic acid occurs through the pathway from ascorbic acid to dehydroascorbic acid (DHA) to diketogulonic acid (the first reaction being reversible and the second irreversible) (Margolis et al., 1990). Therefore, the reported differences between total vitamin C and ascorbic acid concentrations suggest that even though DHA is being produced via ascorbic acid degradation due to UV exposure, as this reaction is reversible, no significant differences are detected in terms of total vitamin C concentration. Contrarily, work published by Koutchma et al. (2002), Tran and Farid (2004), and Tikekar et al. (2011) demonstrated that UV irradiation induces vitamin C degradation in the range of 17 to 40%. Nevertheless, those studies applied treatments using laboratory UV reactors at considerably higher UV

doses (600 mJ·cm^{-2}, 100 mJ·cm^{-2}, and 1.2-1.8 mW·cm^{-2}, respectively), which may cause vitamin C degradation due to heat exposure and other factors. Furthermore, the total vitamin C concentration was considered equivalent to the ascorbic acid concentration while the concentration of DHA was not estimated. In addition, as Tikekar et al. (2011) indicated, the degradation rate of ascorbic acid can be also influenced by other juice physicochemical factors such as pH, organic acid concentrations, and absorbance. These factors help justify in part the differences between previously reported results and the data shown in this study.

The most marked effect of the UV process was evidenced on the concentration of potassium sorbate, characterized by significantly different slopes ($p < 0.0001$) and intercepts ($p = 0.0003$) of the linear relationships between nominal and measured concentrations before and after UV light treatment (Figure 12). As seen, an important degradation of potassium sorbate was produced after the process. Moreover, as a consequence of sorbate degradation, a derivative compound was found in the HPLC chromatogram after UV radiation (Figure 13). This effect was UV-dose dependent (Figure 14) and the presence of the UV-derivative was evidenced by the previously described significant incremental effect of the UV treatment on the juice apparent absorption coefficient ($p = 0.0002$) (Figure 10). Regarding this phenomenon, Cigić et al. (2001) previously found that potassium sorbate isomerizes under UV radiation affecting its antimicrobial activity. Hence, our particular finding demonstrated that this phenomenon could be also observed when liquid food products such as apple juice are exposed to UV even at milder intensities, under shorter exposure times, and using a

commercial UV juice-processing reactor. Nevertheless, further studies are needed to confirm the nature of the derivative compound and the impact of this degradation on the antimicrobial properties of the preservative, specifically in a juice matrix subjected to UV pasteurization.

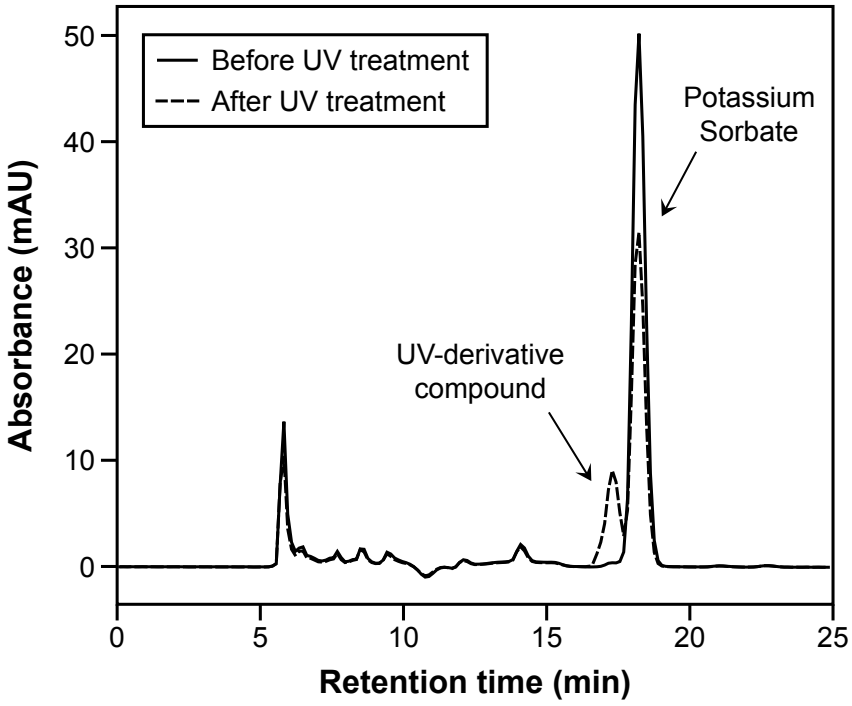

Figure 13. Representative HPLC chromatogram (260 nm) for apple juice containing potassium sorbate at 100 $mg \cdot kg^{-1}$ and treated at 14 $mJ \cdot cm^{-2}$ UV dose.

3.3. Effect of ascorbic acid concentration on the log reduction of *E. coli*

Increments in the concentration of ascorbic acid led to a significant negative effect on the log reduction of *E. coli* ATCC 25922 when UV was applied at a constant flow rate of 214.5 $ml \cdot s^{-1}$ ($p < 0.0001$) and at a fixed UV dose of 14 $mJ \cdot cm^{-2}$ ($p < 0.0001$). Nevertheless, the observed trends varied depending on the conditions at which the treatment was applied (Figure 15). When apple juice was UV treated at a fixed flow rate,

results were in agreement with the findings previously reported by Koutchma et al. (2004) where the inactivation rate of *E. coli* decreased as the solution absorbance increased, and it was inversely proportional to the apparent absorption coefficient.

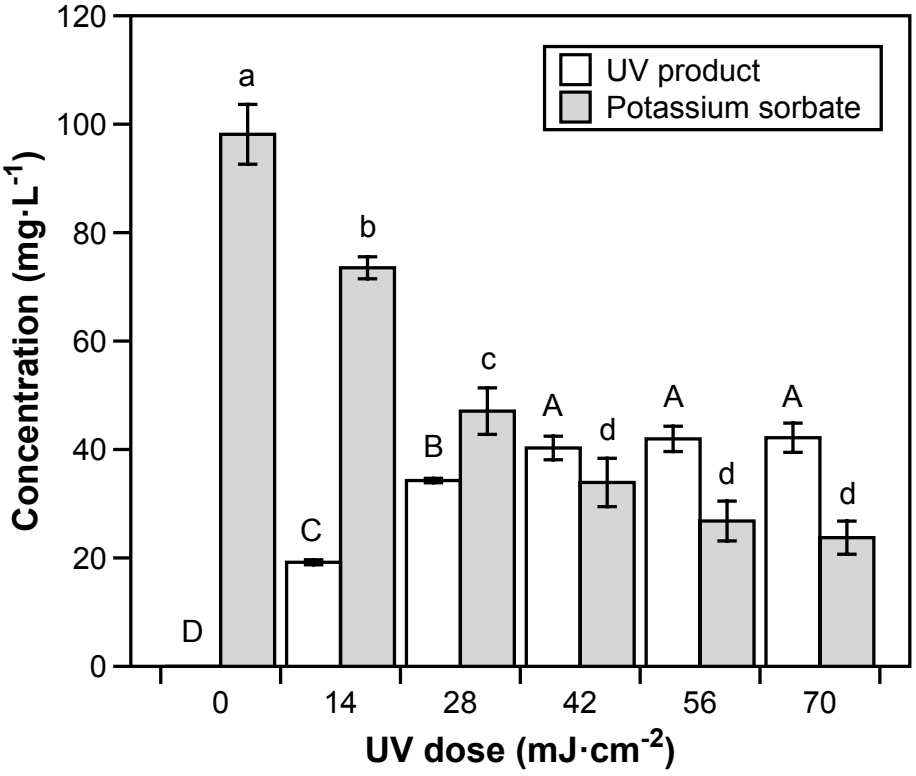

Figure 14. Remaining concentration of potassium sorbate and the derivative UV product as a function of UV exposure. Means with the same lower or uppercase letter are not significantly different (Tukey's test $p > 0.05$) (n = 3, error bars show standard deviation).

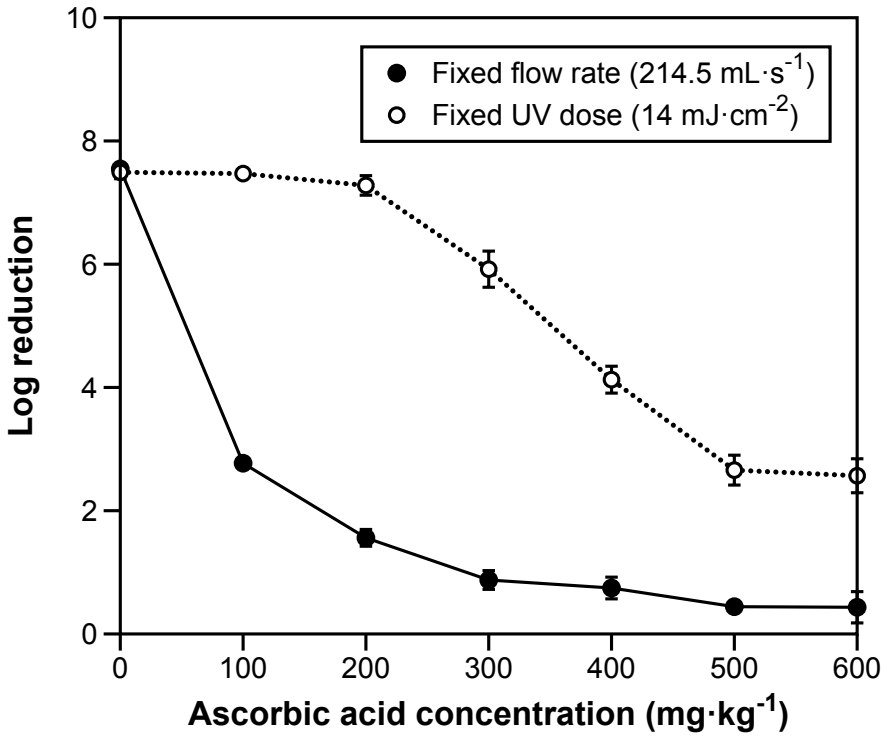

Figure 15. Log reductions of *E. coli* ATCC 25922 in apple juice treated under fixed flow rate and fixed UV dose (n = 3, error bars show standard deviation).

Considering that the U.S. Food and Drug Administration (FDA, 2001) has mandated that juice manufacturers achieve a greater that 5-log reduction of the pertinent pathogen, our results suggest that UV treatments should not be applied under the studied fixed flow rate, because at ascorbic acid concentrations higher than 100 $mg \cdot kg^{-1}$ the achieved reduction was lower than the required microbial inactivation. On the contrary, when UV irradiation was performed at a fixed UV dose of 14 $mJ \cdot cm^{-2}$, the UV treatment guaranteed a higher than 5-log reduction in samples contained between 0 to 300 $mg \cdot kg^{-1}$ of ascorbic acid. With concentrations exceeding 600 $mg \cdot kg^{-1}$ (for the tested juice's absorption coefficient) and due to the automatic flow rate adjustment, the pump was not able to further reduce the flow rate and therefore the machine would stop

processing the juice. Worth noting, under the fixed UV dose setting (which corresponds to the only setting in a commercial *CiderSure* unit), this reactor has been programmed to activate an alarm and stop the process when the validated UV dose of 14 $mJ \cdot cm^{-2}$ is not achieved.

4. CONCLUSIONS

Although the addition of certain compounds represents a viable option to extend the shelf life of UV treated beverages, our study demonstrated that ascorbic acid, sodium benzoate, and potassium sorbate, additives commonly used by the juice industry, increase the juice's absorption coefficient and negatively interfere in the performance of UV treatments. Furthermore, under the studied conditions the UV light application leads to a degradation of ascorbic acid, sulfur dioxide and potassium sorbate, while the addition of ascorbic acid impairs the inactivation of *E. coli* ATCC 25922. Therefore, it is recommended that additives that increase the absorption coefficient of liquid food products or are unfavorably affected by UV should be added after UV pasteurization.

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CHAPTER 6

DETERMINATION OF THE VALIDATION FREQUENCY FOR COMMERCIAL UV JUICE-PROCESSING UNITS

ABSTRACT

The *CiderSure* 3500 is one of the most commonly used UV juice-processing units in the US for the nonthermal processing of apple cider, that fulfills the 5-log performance standard established in the federal juice HACCP regulation. However, the appropriate validation frequency of this machine's quartz tubes is currently unknown by juice processors and regulatory agencies. Presently, an annual validation is recommended by the manufacturer. Historical validation data from 1998 to 2013 of commercially used quartz tubes of the UV processing unit underwent comprehensive statistical analysis. A total of 400 tubes were validated one time, and 212 of those units were revalidated at least once over the evaluated timeframe. Validations were performed at 14 $mJ \cdot cm^{-2}$ UV dose, and under turbulent flow conditions. Every validation showed a greater than 5-log reduction of *Escherichia coli* ATCC 25922, a non-pathogenic surrogate for pathogenic *E. coli* O157:H7, in each of three replicates. For initial validations, a mixed-effects model with log reduction of *E. coli* as response was constructed (400 tubes analyzed in triplicate). The model showed that the year of analysis and the initial inoculum level significantly affected the log reduction of *E. coli* ($P < 0.0001$), which on average was 7.0 ± 0.7. A quadratic relationship between the year of analysis and the response was found. Likewise, for revalidations (212 tubes analyzed in triplicate), the constructed random coefficient model showed that the year of analysis, quadratic effect of year of

analysis and initial inoculum level significantly affected the log reduction of *E. coli* ($P < 0.0001$). For this model, the major source of variance was explained by the year of analysis. The models describe the UV reactor's performance over time and suggest that a validation frequency of every 3 years would be conservatively adequate during the first eight years of use of the quartz tubes. After that and due to the reported quadratic trend, a yearly validation would be recommended.

INTRODUCTION

In 2000, the U.S. Food and Drug Administration (FDA) recognized ultraviolet (UV) light treatment as an alternative for thermal pasteurization of juices and beverages. The requirements state in 21 CFR 179.39, that UV radiation may be safely used for the processing of juice products when the treatment is provided by low-pressure mercury lamps emitting 90% of the emission at a wavelength of 253.7 nanometers, and the juice undergoes turbulent flow through tubes, with a minimum Reynolds number of 2,200 (*25*). Since then, and due to an increased consumer demand for more fresh-like products with enhanced nutritional properties (*2, 23*), the applications of this affordable nonthermal technology have augmented, with the advantage that UV light is characterized by low energy requirements and reduced initial investment in comparison with thermal pasteurization (*4, 15, 18*). Furthermore, with the intention of preventing potentially negative effects, due the application of traditional heat treatments, on the organoleptic properties of cider, and given that unpasteurized and contaminated apple cider has caused several foodborne outbreaks (*6, 21*); many small- and medium-sized cider producers have acquired a commercial UV juice processing unit to safely treat this

beverage. The *CiderSure* 3500 (FPE Inc., Rochester, NY) is one of the most commonly used commercial UV processing machines in the US for the nonthermal processing of apple cider. This machine has been proven effective to ensure more than 5-log reductions of *Escherichia coli* O157:H7 and *Cryptosporidium parvum* in cider (*1, 13*), microorganisms that represent the pertinent pathogens likely to occur in this juice. Therefore, this technology fulfills the 5-log performance standard established in the federal juice HACCP regulation (*24*).

Since 1998, the application of UV light treatments to juices has been actively researched, and in a sixteen year period, a large number of individual quartz tubes used on this UV apparatus have been validated. In the year 2000, 70 of these tubes were subjected to a comprehensive statistical analysis, and the variability within and between tubes, plus the distributions of the mean log reductions of *E. coli* ATCC 25922 and the between-replicate variability were determined (*9*). However, until now, the appropriate frequency of revalidation of these tubes is still unknown by juice processors and regulatory agencies. Presently, an annual validation is recommended by the manufacturer, due to the lack of knowledge regarding the tube variation over time in the commercial use setting. Thus, considering that it is important for the UV-treated cider industry and regulatory agencies to accurately identify the adequate frequency for examining the performance of these quartz tubes, the availability of data corresponding to a large quantity of validations and revalidations represents an exceptional opportunity to determine this frequency, based on a comprehensive statistical approach. The purpose of this study was to statistically analyze historical validation and revalidation

data from 1998 to 2013 of the quartz tubes from commercial UV processing units, with the aim to describe the reactor's performance and its variability over time. We hope that our findings, and consequent recommendation of a validation frequency, will ultimately assist regulatory agencies to establish the most appropriate frequency of revalidation for the commercially available UV juice-processing machine unit used in this study.

MATERIALS AND METHODS

Microbiological analysis. Locally purchased (Geneva, NY) non-pasteurized apple cider, which did not contain any preservatives or other additives, was inoculated with *Escherichia* coli ATCC 25922, a clinical isolate from the American Type Culture Collection, and a non-pathogenic surrogate that has shown similar UV sensitivity to *E. coli* O157:H7 (*20*). A single isolated colony of *E. coli* grown on Trypticase soy agar (TSA) (Difco, Becton Dickinson, Sparks, MD) was transferred in 10 ml of Trypticase soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD) and incubated for 5 ± 1 h at 35 ± 2ºC. The inoculum was then transferred into 400 ml of TSB and incubated for 20 ± 2 h at 35 ± 2ºC to stationary phase in an Innova 2300 rotary platform shaker (New Brunswick Scientific Co., Edison, NJ) at 250 rpm. Prior to the validation of the quartz tubes, approximately 1.8 l of cider was inoculated with a 20 ml aliquot of the *E. coli* inoculum. An initial concentration of 6 to 7-log $CFU \cdot ml^{-1}$ of the surrogate was targeted. Inoculated ciders were aseptically sampled before and after UV processing, and analyzed immediately. For UV-treated samples, 1 ml of cider and two serial dilutions in sterile 0.1% peptone water were aseptically plated (in duplicate) in Petri dishes, to which approximately 20 ml of sterilized TSA (Difco, Becton Dickinson, Sparks, MD) was

pour-plated and mixed thoroughly. For untreated samples, six serial dilutions in sterile 0.1% peptone water were required. Petri dishes were incubated for 20 ± 2 h at 35 ± 2ºC before enumeration, and the level of microbial reduction was calculated as log (*N/No*) where *N* refers to the after treatment *E. coli* count, and *No* to the initial count, both in $CFU \cdot ml^{-1}$. Each quartz tube was evaluated in triplicate.

UV juice-processing unit. Apple cider was run through a commercial *CiderSure* 3500 UV juice processing unit (FPE Inc., Rochester, NY) at a wavelength of 254 nm, and guaranteeing a turbulent flow regime and a constant UV dose of 14 $mJ \cdot cm^{-2}$. The UV processing unit is comprised of a stainless steel housing and an inner quartz tube. The apple cider is pumped between the outer steel housing and inner quartz tube using a positive displacement pump, and the product is exposed to eight germicidal low-pressure mercury lamps placed concentrically within the interior of the quartz-stainless steel cylinder (*1*). Two UV light sensors, located at the bottom and top of the outer cylinder, measure the UV light transmittance through the cider every 50 ms. Based on the product transmittance measurements, this machine has been programmed to automatically adjust the pump flow rate ensuring a constant UV dose exposure throughout the UV process (*1, 20*). This apparatus has been provided with an automatic system designed to shut down the process if a UV light sensor fails or if the sensor indicates that a minimum of 14 $mJ \cdot cm^{-2}$ UV dose, which represents the critical limit on the application of this treatment, has not been met.

Validations and revalidations. Throughout sixteen consecutive years (from 1998 to 2013), a total of 400 quartz tubes were validated at least one time, and 212 of those same tubes have been revalidated at least once. All quartz tubes were brought to the Food Microbiology Laboratory at the New York State Agricultural Experiment Station (Geneva, NY) and subjected to the standard validation procedure designed by this laboratory and described above. During the evaluated timeframe, all quartz tubes were validated and revalidated by the same analyst, and using the same UV juice-processing unit, which was comprised of a pump, UV light sensors, software, and eight germicidal UV lamps. Over the years, regular maintenance has been provided to the UV unit, which includes repairs to the pump, and replacement of the UV light sensors and lamps.

Statistical analyses

The mixed-effects and random coefficient models were constructed and analyzed using JMP® version 11 (SAS Institute Inc., Cary, NC). Effects were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Initial tube validations

All initial validations showed greater than 5-log reductions of *E. coli* ATCC 25922 in each of the three replicates. This result is explained by the fact that if a tube failed to achieve the minimum 5-log pathogen reduction during the first validation, that tube was not sold to the cider industry, and therefore it was not considered for further statistical analysis. Prior to this study, it was found that the risk of obtaining a less than 100,000-

fold reduction of *E. coli*, by using these UV light-processing tubes for treating apple cider, occurs less than 0.2% of the time (*9*).

For the analysis of the initial validations, a mixed-effects model with log reduction of *E. coli* as response was constructed. As stated by Wang et al. (*26*), a mixed-effects model can specify a realistic model for the correlation existing between repeated measurements, which in this case were represented by the logarithmic reduction of *E. coli* due to the UV light exposure, measured in the same tube on three occasions. The results associated with 400 tubes, analyzed in triplicate, and evaluated between 1998 and 2013 gave a total of 1200 observations. The variables of initial *E. coli* count, year of analysis (with year zero corresponding to 1998), and a quadratic effect of year of analysis were added to the model as continuous fixed effects, while the tube ID was included as a random effect.

An average of 7.0 ± 0.7 log reductions of *E. coli* was obtained, and as observed in Figure 16, the histogram of log reduction shows a skew to the left with a minimum value of 5.01 log reduction, boundary at which the data set was artificially truncated when the tubes that obtained a lower than 5-log reduction were not considered for further analysis. Additionally, the upper limit was found at 8.29 log reduction. Worth noting, this value may not reflect the maximum effectiveness of the apparatus. Instead, it suggests that the efficacy of these UV processing quartz tubes could be limited, in part, by the initial and targeted *E. coli* concentration in the test cider. Likewise, using a subset of this data (70 tubes), Duffy et al. (*9*) previously reported high and low log reduction tails when

@RISK and Analytica simulations were used to model the data's distribution, indicating that those tails are probably an accurate reflection of the UV processing units' performance rather than an artifact of simulation.

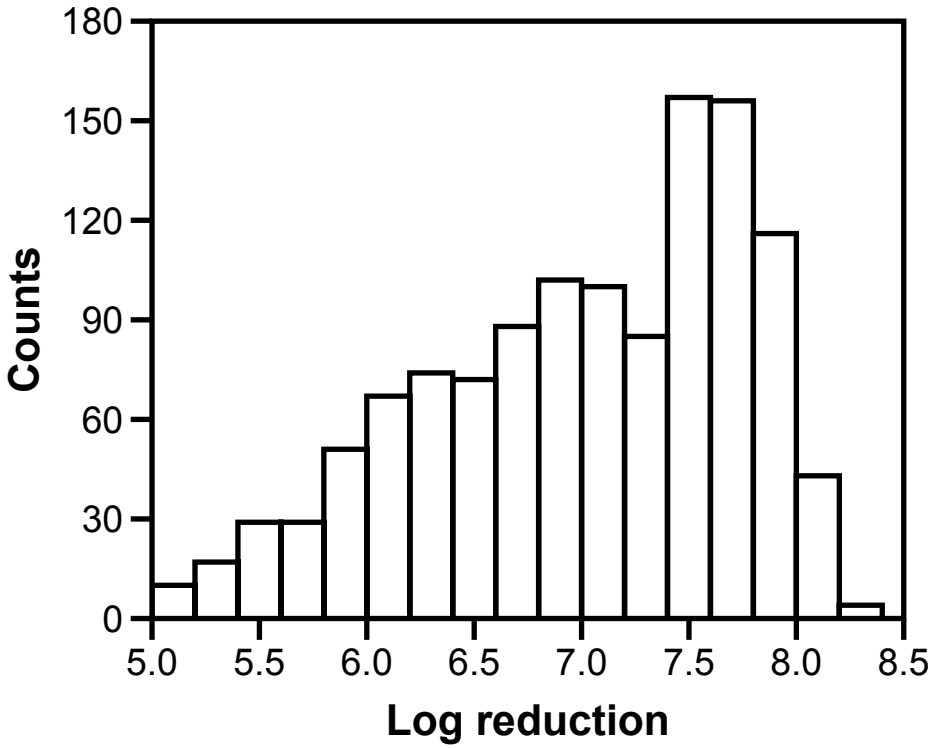

Figure 16. Histogram of log reduction of *E. coli* ATCC 25922 in apple cider subjected to UV treatment at 14 $mJ \cdot cm^{-2}$ UV dose by using a commercial UV juice processing reactor, corresponding to validation trials (n=1200).

The constructed mixed-effects model showed that the effects of initial *E. coli* count and year of analysis significantly affected the log reduction of *E. coli* ($P < 0.0001$) (Table 13), and the relationship between the response variable and the year of analysis was found to be quadratic, with an increasing and then diminishing effect over time. Moreover, the model showed that the variance between the quartz tubes was considerably higher, representing 70% of the total variance, in comparison with the variance observed within tubes (Table 14).

Table 13. Parameters estimates of the fixed effects included in the mixed-effects model used to analyze the initial validations of the quartz tubes with log reduction of *E. coli* ATCC 25922 as response.

Term	Estimate	Standard error	*P* > |t|
Intercept	0.6	0.6	0.3542
Initial *E. coli* count	0.74	0.08	<0.0001
Year of analysis	0.22	0.03	<0.0001
(Year of analysis)*(Year of analysis)	-0.011	0.002	<0.0001

Tube revalidations

With the objective of establishing the appropriate frequency of validation of the quartz tubes, revalidation data from 1998 to 2013 underwent a comprehensive statistical analysis. The results of 212 UV processing quartz tubes (each analyzed in triplicate) and revalidated at least once (more than two validations were conducted) over the selected timeframe (a total of 1740 observations), were used to construct a random coefficient model with random intercept and log reduction of *E. coli* as response. According to Cudeck and Harring (*5*), in the context of repeated measure studies, the random coefficients models are based on the idea that the process of change is defined for each unit of study, in this case each quartz tube, yet also is related to the population mean trajectory over time. The variables of initial *E. coli* count, year of analysis (with year zero corresponding to 1998), and a quadratic effect of year of analysis, were considered as the fixed effects, while the tube ID, and the year of analysis (as a nominal variable and nested within tube ID), were added as the random effects.

Table 14. Variance components of the random effects included in the mixed-effects model used to analyze the initial validations of the quartz tubes with log reduction of *E. coli* ATCC 25922 as response.

Random effect	Variance component	Percentage
Tube ID	0.30	70
Residual	0.13	30
Total	0.43	100

The parameters estimates of the constructed model are given in Table 15. As observed, the initial *E. coli* count and year of analysis significantly affected the log reduction of *E. coli* ($P < 0.0001$). Once again, a quadratic behavior of year of analysis with an increasing and then diminishing effect on the log reduction of *E. coli* over time was found. Considering the overall mean initial count of 7.57 logs of *E. coli*, it was found that the maximum predicted value of *E. coli* reduction as a response, which corresponds to 8.00 logs, was observed at 8.86 years after the first validation. After this time, the bacterial reduction levels begin to decrease. The residual unexplained variance within tubes was 29%, whereas the variance between tubes caused only 5% of the total variance. The rest –and most– of the variance was explained by the random effect of year of analysis (Table 16). The higher level of variance caused by the year of analysis is likely due to the expected and widely reported differences of the physicochemical characteristics of the apple ciders used over the 16 year period. Although a slight reduction of *E. coli* in the juices (without subjecting the beverage to the UV radiation) is possible, the effect was not deemed substantial. The chemical and nutritional composition of apple cider has been determined in several studies for different apple varieties, and significant differences between apple cultivars have been consistently

reported (*1, 3, 10, 12, 14, 17, 19*). Also, variables such as the growing season and storage conditions represent some of the parameters that influence the physicochemical properties of fruits and therefore ciders. Worth noting, it has been also demonstrated that these differences between the physicochemical properties of apple ciders may affect the survival of *E. coli* during the application of UV light treatments, due to potential differentiated antimicrobial advantages and disadvantages provided by the juice (*1*). Interestingly, it has been reported that apple cider produced from stored apples shows less inhibition of *E. coli* O157:H7 than that made with freshly harvested apples (*7, 8, 22*). This may explain in part the observed variability among the revalidations performed in different years, and therefore using different ciders.

Table 15. Parameters estimates of the fixed effects included in the random coefficient model used to analyze the revalidations of the quartz tubes with log reduction of *E. coli* ATCC 25922 as response.

Term	Estimate	Standard error	*P* > |t|
Intercept	0.5	0.5	0.3113
Initial *E. coli* count	0.74	0.07	<0.0001
Year of analysis	0.23	0.03	<0.0001
(Year of analysis)*(Year of analysis)	-0.013	0.002	<0.0001

Nonetheless, considering exclusively the revalidation data, a greater than 5-log reduction of *E. coli* ATCC 25922 was observed for all the tubes tested, and on each of the three corresponding replicates. This result suggests that although the differences among ciders are important, and probably explain most of the variance of the resulting

model, the inclusion of new apple varieties to produce cider, and the normal differences between the physicochemical properties of the fruits did not compromise the performance of the tubes during the studied timeframe. Therefore, it is the author's opinion that this source and magnitude of variability does not justify performing yearly revalidations, as it is currently recommended by the manufacturer.

Table 16. Variance components of the random effects included in the random coefficient model used to analyze the revalidations of the quartz tubes with log reduction of *E. coli* ATCC 25922 as response.

Random effect	Variance component	Percentage
Tube ID	0.02	5
Year of analysis	0.28	66
Residual	0.12	29
Total	0.42	100

Different elements may explain the quadratic trend found with the random coefficient model. A loss of the UV sensitivity of sensors, lamp degradation, and darkening of the quartz caused by cider contact over the years, besides other changes in the quartz tubes that have not been completely understood, could be the cause of the observed results. For instance, if UV light sensors are not recalibrated, they may loose their UV transmittance sensitivity over time, thus slowing the flow rate of cider through the reactor and therefore causing extended times of UV light exposure that ultimately translate into higher microbial reductions. A reduction of the germicidal capacity of the UV lamps is also expected over time. The aging of lamps influences the emitted UV energy intensity, and is primarily caused by two factors: solarization of the lamp wall

material, and blackening due to deposits of sputtered oxides from the electrodes (16). The content within the lamps is continuously exposed to changes in pressure and temperature, thus the electrodes inside decay and deposit material on the interior quartz, reducing the lamps output. Moreover, the efficacy of the lamp is directly related to the saturated mercury pressure inside the lamp and many parts on the UV lamps such as the glass bulb, quartz bulb, electrode emitter, and metal parts consume mercury during the lamp's life and reduce its efficacy (*11*). Worth noting, these effects are expected to be controlled to some extent by the UV light sensors and their effect on the apparatus' pump, which in turn affects the flow of cider through the quartz tubes.

The constructed models reported in this study describe the quartz tubes' performance over time, and as indicated by Duffy et al. (*9*), quantifying the variability of a food process could be in extreme useful to formulate better predictive models and also to separate the uncertainty from variables as much as possible. This study aims to help regulatory agencies to establish the appropriate frequency of validation for the quartz tubes of a commercial UV processing unit, and based on the constructed random coefficient model, we recommend revalidating the quartz tubes every three years during the first eight years of use. After that, and due to the reported quadratic trend –which predicts the *E. coli* reduction to decrease after 8.86 years–, a yearly validation is recommended instead. We also suggest that any changes in the UV juice processing unit that may negatively compromise the performance of the reactor, and ultimately the safety of the cider, such as changes in the computer and software, or replacement of the pump, UV light sensors, and mercury lamps should be followed by the application of

the validation procedure of the quartz tubes, regardless of the time and results associated to the last validation performed. Likewise, the use of this UV machine for treating different juices or beverages, and changes in the formulation of already validated liquid products, must be subjected to the validation protocol before launching the beverage to market. Furthermore, it is important to clarify that the frequency of validation suggested in this study does not substitute the regular verification procedures, which include periodical maintenance of UV light sensors, pump and computer, and the standard cleaning and sanitizing protocols of the UV unit recommended by the manufacturer.

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CHAPTER 7

CONCLUSIONS AND FUTURE WORK

Thermal tolerance and survival of pathogenic microorganisms relevant to juices

Data from the study summarized in Chapter 2 will be useful as a reference for establishing the critical limits for the safe thermal processing of pH controlled juices and similar products. However, it is important to consider that the application of the processing conditions (time and temperature of pasteurization) extrapolated from our study, should be validated before their implementation in commercial applications. These safety validations should be performed in terms of efficacy of the recommended treatments against other pathogenic microorganisms of public health significance in juices, such as *Cryptosporidium parvum* in apple juice, apple cider and apple-carrot juice blends; and *Salmonella* in citrus juices. Moreover, it would be interesting to evaluate if consumers prefer or can detect significant differences among juices and beverages treated with a traditional flash pasteurization protocol (71ºC for 6 s) or subjected to a milder heat treatment such as the recommended in Chapter 2.

The determination of the thermal tolerance parameters of *E. coli* O157:H7 described in Chapter 2 was performed using only one strain (C7927) and, as demonstrated in Chapter 3, significant differences on the thermal tolerance and survival among strains are expected. Thus, further investigation is required to validate if the heat treatments suggested in Chapter 2 will be sufficient against other O157:H7 strains. This can be

performed using a cocktail of at least five *E. coli* strains, as recommended in the third chapter of this dissertation.

Additionally, the data reported in Chapter 2 would be useful for process authorities to establish adequate thermal treatment protocols for refrigerated sauces, dressing, soups and other liquid food products with a pH below 4.6 and acidified with different organic acids. Nonetheless, the influence of other physicochemical properties of these products including soluble solids, insoluble solids and fat content, as well as the presence of natural antimicrobial compounds, on the thermal tolerance and survival responses of the pathogens relevant to those products should be evaluated.

Thermal tolerance and survival enhanced responses of *E. coli* O157:H7 and O111

As demonstrated by the results reported in Chapters 2 and 3, inducing an acid shock or acid adaptation in Shiga toxin-producing *E. coli* strains enhances the thermal tolerance and survival of these pathogens in apple juice. Also, it has been reported in the literature that this exposure to moderate acidic environment induces a cross-protection against other environmental stresses that may be encountered during food processing, including salt, activated lactoperoxidase system, surface-active agents, radiation, and others (Leyer et al., 1995). Thus, considering the lack of evidence in the literature, it would be interesting to investigate if the acid shock and acid adaptation have also a significant effect on the UV tolerance and survival of Shiga toxin-producing *E. coli* in juices when a UV treatment is applied under the conditions recommended by the FDA (FDA, 2013) and using a commercial UV unit such as the *CiderSure.* Additionally,

further investigation is needed to elucidate if there is an effect of acid adaptation and acid shock on the tolerance and survival of pathogens in juices treated with other emergent technologies such as high pressure processing, pulse electric fields and plasma. Also, it would be interesting to study if an enhanced tolerance response to adverse environments would be observed for other foodborne pathogens including *Salmonella,* and *Listeria monocytogenes*, and spoilage microorganisms subjected to these emerging nonthermal technologies.

Additionally, is necessary to conduct research regarding the changes in gene expression and membrane composition of Shiga toxin-producing *E. coli* when exposed to acid adaptation and acid shock protocols. Microarrays, RNA sequencing, and proteomics are some of the technological approaches that can be used for this purpose.

Application of UV light treatments to juices different than apple juice and cider

Further investigation into the effectiveness of this technological approach for the safe treatment of juices (other than apple juice and cider) such as watermelon, pineapple, white grape juices and coconut water is of interest to the juice industry. For this purpose, microbial challenge studies oriented to evaluate the efficacy of UV light technology against the pathogens of concern likely to occur in these juices should be executed. If results indicate that the treatment is sufficient to ensure the safety of these products, further work would be required to evaluate the shelf life of these beverages in comparison with the shelf life of the heat pasteurized counterparts. Moreover, the physicochemical and nutritional properties of the UV treated should be compared

against pasteurized juices, as well as consumers' potential preference for the more "fresh-like" versions.

Coupling of UV light with other thermal and nonthermal technologies

Considering that UV treated juices and juice drinks are not shelf stable products and that some physicochemical properties of juices, such as those described in Chapter 4, may negatively affect the performance of this technology, it would be important to investigate the feasibility and advantages of coupling UV light with traditional approaches such as heat treatments, as well as with other emerging nonthermal technologies. This research should contemplate microbial validations to confirm that a safe product would be obtained, as well as a comprehensive evaluation of other factors relevant to the juice industry such as the cost of the hurdle treatment, changes in nutritional and physicochemical properties and shelf life of the resulting juice. Although some research efforts of coupling UV light with other technologies including pulsed electric fields (PEF), radio frequency electric fields (RFEF), manothermosonication, and high intensity ultrasound (HIU) have been executed and published in the last decade (Gachovska et al., 2008; Martin-Belloso and Sobrino-Lopez, 2011; Noci et al., 2008; Walkling-Ribeiro et al., 2008), further research is needed to optimize the combination of these technologies and to offer efficient, sustainable, cost-effective and affordable solutions to the food industry.

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